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Design, synthesis and biological evaluation of 1,5-disubstituted α-amino tetrazole derivatives as non-covalent inflammasome-caspase-1 complex inhibitors with potential application against immune and inflammatory disorders

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ABSTRACT

Compounds targeting the inflammasome-caspase-1 pathway could be of use for the treatment of inflammation and inflammatory diseases. Previous caspase-1 inhibitors were in great majority covalent inhibitors and failed in clinical trials. Using a mixed modelling, computational screening, synthesis and in vitro testing approach, we identified a novel class of non-covalent caspase-1 non cytotoxic inhibitors which are able to inhibit IL-1β release in activated macrophages in the low μM range, in line with the best activities observed for the known covalent inhibitors. Our compounds could form the basis of further optimization towards potent drugs for the treatment of inflammation and inflammatory disorders including also dysregulated inflammation in Covid 19.

1. Introduction

Inflammasomes are multiprotein complexes emerged as key regulators of innate immune response and inflammation [1]. Their assembly in cytosol, in response to molecules derived from microorganism (pathogen-associated molecular patterns - PAMPs) or endogenous danger signals (damage-associated molecular patterns - DAMPs), induces the activation of the holoenzyme inflammasome-caspase 1 complex, that triggers the release of the mature form of inteleukin-1β (IL-1β) and IL-18 and drives pyroptosis. The release in the cytosol of these potent pro-inflammatory mediators, culminate in beneficial immune responses and antimicrobial defense [2]. However, a deregulated activation and secretion of inflammatory mediators induced by endogenous danger signals, is linked to the onset or progression of cardiovascular diseases, inflammatory pathologies, neurodegenerative, metabolic and autoimmune diseases and cancer [3–10]. There is a growing evidence on the relation between innate immunity, excessive release of pro-inflammatory IL-1β and various immune and inflammatory disorders, including CNS diseases such as Alzheimer's (AD), Parkinson's (PD) and Huntington's (HD) diseases, amyotrophic lateral sclerosis (ALS), and multiple sclerosis (MS) [11–15]. Activation of microglia and other cell types in the brain, in response to DAMPs in the form of misfolded proteins, mislocalized nucleic acids or aggregated peptides such as amyloid-β (Aβ) for AD, α-synuclein for PD, superoxide dismutase for ALS and huntingtin for HD, leading to uncontrolled release of pro-inflammatory mediators, is emerged as a key mechanism in the development and progression of major neurodegenerative disease [16–18]. Moreover, there is also a

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growing evidence that the pathway inflammasome NLRP3/Caspase-1 is overactivated by the SARS-Cov-2 and may be responsible for the high mortality observed in the Covid-19 patients due to the inflammatory internal organs collapse driven by the cytokine storm induced by the virus [19–23]. It is now quite clear that an effective pharmacological approach to Covid-19 must comprise an antiviral drug in combination with an inflammation modulator able to regulate the innate immunity system response preserving its regular activity but quenching its overactivation.

Caspase-1 activity is essential in immune and inflammatory response irrespective of molecular signals that induce the assembly of the different holoenzyme inflammasome-caspase-1 complexes. In addition to pro-IL-1β and pro-IL18, also gasdermin D (GSDMD) is a recognized substrate for caspase-1 to generate an N-terminal cleavage product (GSDMD-NT) that induces plasma membrane pores and pyroptosis [24]. Recent studies have shed some light on the nature of the caspase-1 active species (p33/p10 linked to inflammasome to form the inflammasome-caspase-1 complex), on the location of caspase-1 activity, on the intrinsic time limiting mechanism of caspase-1 activity of the inflammasome-dependent inflammatory responses, as well as on the role of caspase-1 in inducing pyroptosis by mediating GSDMD cleavage [24,25]. Compounds inhibiting caspase-1 have been proposed as promising new therapeutics for the treatment of inflammation driven diseases, and many potent caspase-1 inhibitors have been developed, and have demonstrated their effect in preclinical studies for different indications, but only few compounds have progressed in clinical trials failing to reach the desired clinical endpoints (Fig. 1) [26–38].

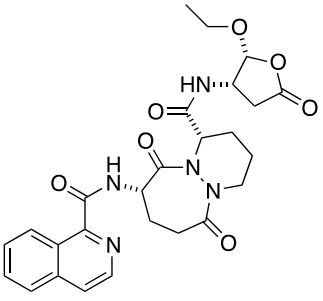
All these peptidomimetic molecules and the currently used peptidic caspase-1 tool compounds are often unselective and covalent as mode of action (MoA), peptidic in nature, and with very low blood-brain barrier (BBB) penetration, if at all. The lack of selectivity and the off-target interaction with other nucleophiles in vivo due to their covalent MoA, may have contributed to the effects observed in long term toxicity studies in dogs accompanying the human phase II study of Pralnacasan (VX-740) [39]. Novel improved compounds are urgently needed for testing the hypothesis if suitable caspase-1 inhibitors are able to slow down or prevent inflammatory diseases in humans also because there are no caspase-1 inhibitor drugs approved for clinical use on the market. Here we report the design and synthesis of a new class of potent and stable non-peptidomimetic and non-covalent caspase-1 inhibitors designed to overcome the drawbacks of the previous compounds for therapeutic applications.

2. Results and discussion

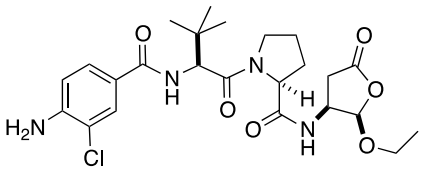
We have designed a new class of non-covalent, non-peptidic, small molecule caspase-1 inhibitors by structure-based drug design. Our goal was to obtain a new class of stable and bioavailable inhibitors rationally designed to interact with the catalytic site of the enzyme in a competitive and non-covalent MoA in order to avoid off-target interactions with other nucleophiles leading to side and toxic effects previously described in clinical trials for covalent inhibitors. The multicomponent reaction (MCR) approach was used for the synthesis of our molecules, because it offers a significant advantage over conventional linear step synthesis permitting the one pot assembly of very complex structures and the easy synthesis of a collection of derivatives.

2.1. Design

Our design of non-covalent caspase-1 inhibitors is based on a substrate mimicry approach (Fig. 2). The WEAD (trp-glu-ala-asp) sequence is generally recognized by caspase-1 and target proteins are always aspartyl-cleaved (P1=Asp) [40]. Thus, we aimed to design non cleavable aspartyl mimicking scaffolds which would allow for easy modification in further positions to address P2–P4 sites. Multicomponent reaction chemistry is known to address a large drug-like scaffold space and is compatible to multiple functional (often unprotected) groups [41–43]. For example, MCR has been recently used for the improved synthesis of the drugs atorvastatin, praziquantel and ivosidenib [44–46]. Multicomponent reaction chemistry allows for the simultaneous introduction of several side chains via different building blocks which potentially can mimic P2–P4 sites. Multicomponent reactions we are experienced in are isocyanide-based MCRs [47]. Specifically, tetrazole yielding MCRs recently attracted considerable attention due to their scaffold diversity, ease of access and drug-like properties [48]. Thus, we decided to investigate the Ugi tetrazole variation (UT-4CR) and search the UT-4CR chemical space for potential caspase-1 inhibitors. The UT-4CR variation consists of the reaction of an isocyanide with an oxo component and a secondary or primary amine as variable building blocks to yield 1,5-disubstituted α-amino tetrazole [49,50]. Our design includes a 4-amino-3-hydroxy butanoic acid mimicking the aspartyl P1 side chain. Conformational analysis of the UT-4CR scaffolds suggests the introduction of the 4-amino-3-hydroxy butanoic acid moiety as an amine component. The butanoic acid is designed as the main Asp mimicking part with the



VX740 pralnacasan (prodrug)
VRT-18858 - inhibitor of caspase-1 (IC_{50} 1.3 nM) and
LPS-induced IL-1β release in vitro PBMCs (IC_{50} 0.85 μM)



VX-765 belnacasan (prodrug)
VRT-043198 - inhibitor of caspase-1 (Ki 0.8 nM) and
LPS-induced IL-1β release in vitro PBMCs (IC_{50} 0.47 μM)

Fig. 1. Some caspase-1 inhibitors progressed in clinical trials.

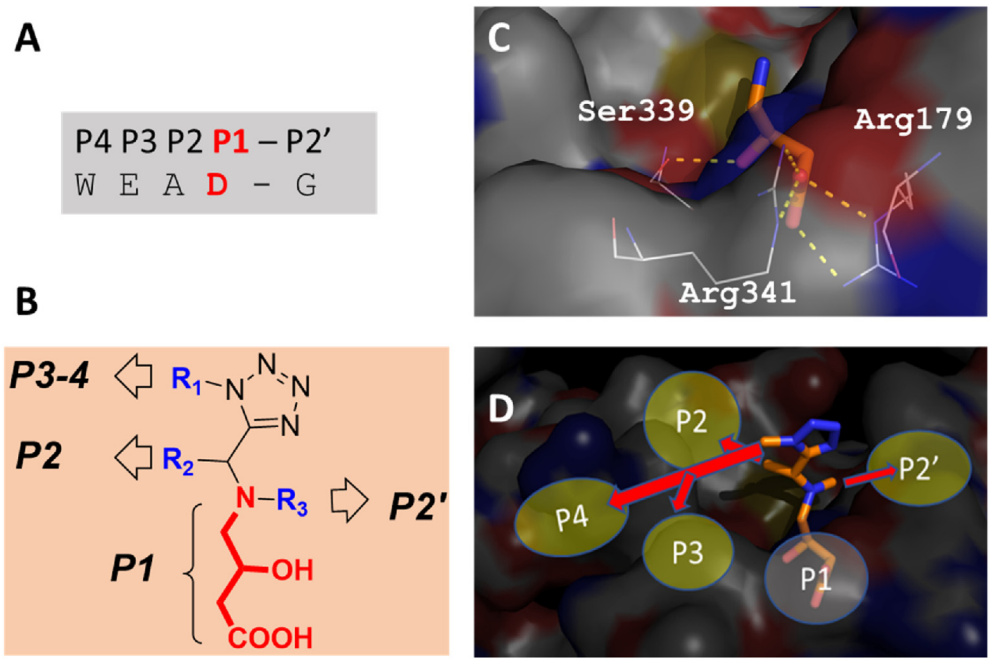


Fig. 2. Caspase-1 inhibitor design. A: peptidic recognized sequence; B: non cleavable aspartyl mimicking scaffolds; C: charge-charge and hydrogen bonding interactions of butanoic acid residue; D: P2–P4 and P2′ possible addressed sites.

carboxylic acid undergoing multiple charge-charge and hydrogen bonding interactions to Arg179, Arg341 and Gln283. The 3-hydroxy group in our design would undergo an additional hydrogen bonding contact to Ser339 backbone carbonyl-O in the polar Asp pocket. The oxo component could address the P2 site and the isocyanide component the P3–P4 sites. Additionally, N-substitution (alkylation, acylation) of the 4-amino-2-hydroxy butanoic acid moiety could potentially address the P2' site.

We pursued a computational prescreening to decide which compounds to synthesize and test. The ChemAxon suite was used in order to create a virtual library of compounds based on multicomponent reaction (MCR) chemistry [51]. Babel and Moloc molecular design software were used to generate 3D conformations, to fix the unique molecular library to a fragment and to optimize the energy and the overlap of the library with the protein [52]. As the receptor for modelling we were using the 2HBQ PDB structure [53]. It is a crystal structure of wildtype human caspase-1 in complex with covalent 3-[2-(2-benzyloxycarbonylamino-3-methyl-butyrylamino)-propionylamino]-4-oxo-pentanoic acid (z-VAD-FMK) inhibitor. A typical result from virtual screening the UT-4CR chemical space is shown in Fig. 3.

2.2. Synthetic routes

In order to explore the UT-4CR chemical space for potential caspase-1 inhibitors the series of the selected 1,5-disubstituted α-amino tetrazole **5** and **16** were synthesized via MCRs. The four components of the UT-4CR needed to meet all the requirements established by the computational study were methyl-4-amino-3-hydroxybutanoate as the amine component **1**, an aliphatic or aromatic aldehyde **2**, a 4-isocyanobutanamide derivative **3**, and trimethylsilyl azide **4** (Scheme 1). A collection of derivatives **5** and **16** has been then synthesized exploiting variations to the aldehyde and isocyanide components of the MCR.

The oxo component (R_2-CHO) **2** was chosen from eight commercially available aldehydes to address the hydrophobic pocket P2 by an aliphatic or aromatic R_2 residue. To address P3–P4 sites, three isocyanide derivatives (component **3**) equipped with different aromatic residue were synthesized in high yields starting from 4-aminobutanoic acid (Scheme 2). The reaction of the amino acid **6** with propyl formate at 90 °C gave compound **7** in quantitative yield that was coupled with the three different amines **8**, **9** and **10** to give compounds **11**, **12** and **13**. Isocyanides **3a-c** were then synthesized in good yield ranging from 85% to 97% via dehydration of N-formyl derivatives. The amine component for the MCRs, methyl-4-amino-3-hydroxybutanoate **1** mimic of the aspartic residue, was quantitatively obtained *via* an easy esterification of the corresponding acid.

1,5-Disubstituted α-amino tetrazole derivatives **5aa-5ah**, **5ba-5bf** and **5ca-5ce** were synthesized with good yields via reaction of isocyanides **3a-c**, aldehydes **2**, amine **1** and trimethylsilyl azide **4** in methanol at room temperature and subsequent basic hydrolysis of the ester obtained (Scheme 3). The reactions were performed by using racemic amine component **1** and because a second stereocentre was generated during the reaction, the tetrazole derivatives were obtained as a diastereomeric mixture of couples of enantiomers. In order to verify the effect of the chirality of carbon 3 stereocentre on the enzymatic inhibition activity some MCRs were repeated by using commercially available enantiopure (*R*)- or (*S*)-methyl-4-amino-3-hydroxybutanoate **1** to give (3*R*) or (3*S*) diastereomeric mixture of 1,5-disubstituted α-amino tetrazole derivatives **5**.

Finally, in order to address the P2′ site, the amine component 4-amino-3-hydroxybutanoate **1** was N-alkylated via reductive amination to give the secondary amine **15** that was used in the MCRs (Scheme 4). The MCRs were performed by using the isocyanide **3a** containing the tetrahydroisoquinoline moiety that showed to induce the best enzymatic activity with respect to the other isocyanides **3b** and **3c** (see below). Compounds **16aa-16ad** were obtained in good yields after basic hydrolysis and purification on a silica gel pad (Scheme 4).

The chemical structures of the 1,5-disubstituted α-amino tetrazole derivatives **5** and **16** were characterized by ^{1}H and ^{13}C NMR. The ^{1}H and ^{13}C NMR spectra of these compounds showed splitting

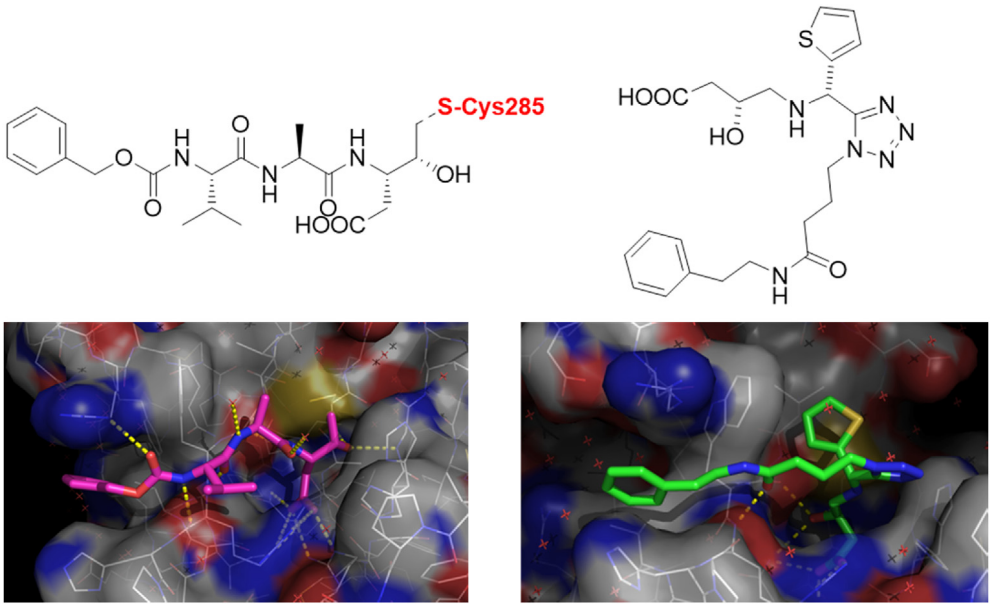
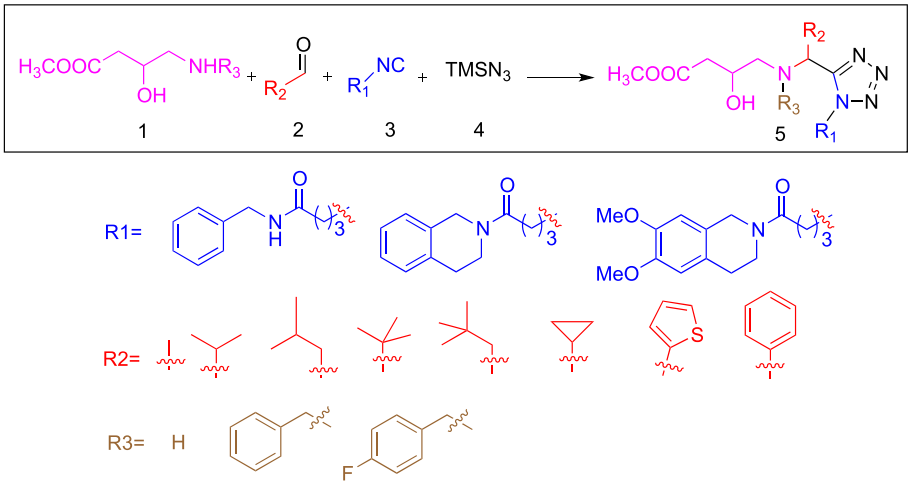


Fig. 3. Result of the virtual screening for a selected UT-4CR compound.



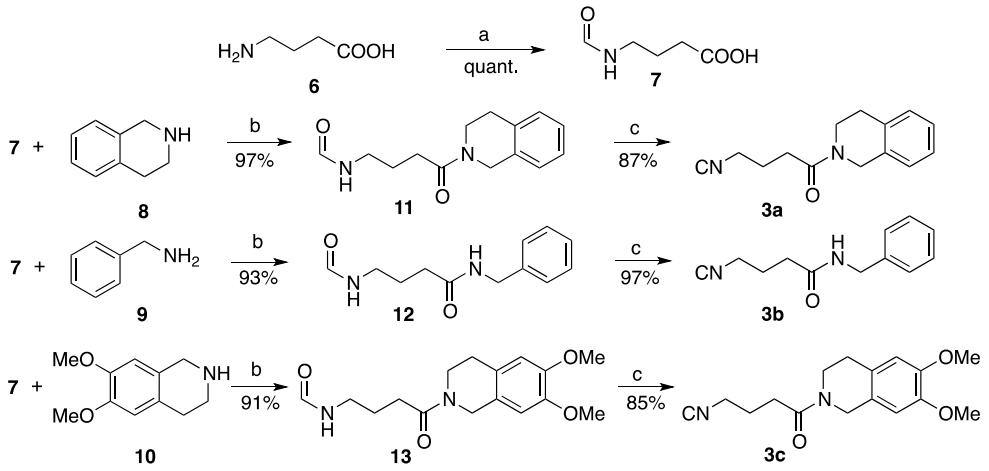
Scheme 1. MCR synthesis of the target molecules.

signals due to the presence of a mixture of atropoisomers. ^{1}H NMR spectra exhibited a characteristic multiplet signal around δ 4.5−4.6 ppm relative to the methylene group (CH_2) bonded to the N of the tetrazole ring and a multiplet signal around δ 4.38 ppm for the proton adjacent to aliphatic R_2 substituent. A carbon signal characteristic of the methylene group adjacent to the tetrazole ring were observed around δ 45.3.

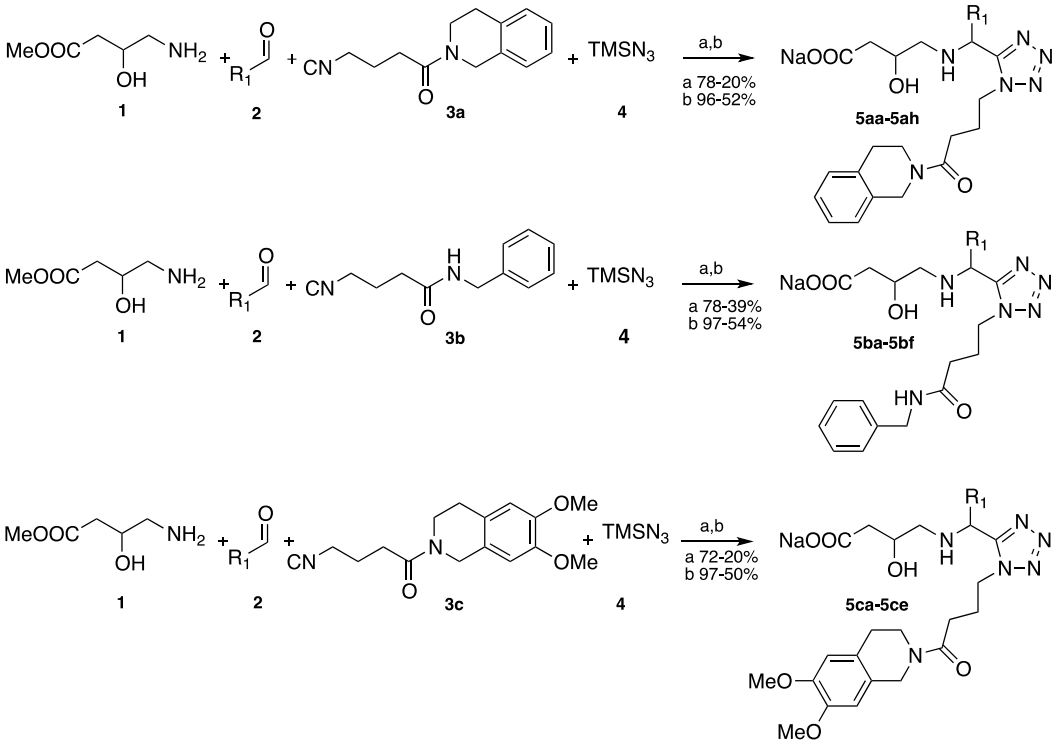
2.3. Caspase-1 inhibition activity

Compounds **5** and **16** were monitored as a time-course measurement of the increase in fluorescence signal from fluorescently labelled peptide substrate. The inhibition activities of the 1,5-disubstituted α-amino tetrazole derivatives **5aa-5ah** containing the tetrahydroisoquinoline moiety, **5ba-5bf** containing the benzyl moiety and **5ca-ce** containing the 6,7-dimethoxy-tetrahydroisoquinoline moiety are reported in Table 1, Table 2 and Table 3 respectively as IC_{50} values and percent of enzymatic activity at 100 μM concentration.

Comparing the activity of the three series of compounds, the better potency was observed for 1,5-disubstituted α-amino tetrazole **5aa-5ah**, showing that the tetrahydroisoquinoline moiety determines a better P3−P4 sites interactions with respect to benzyl or 6,7-dimethoxy-tetrahydroisoquinoline. Tuning of the hydrophobic P2 site interactions by aliphatic and aromatic R_2 residues gave tetrazole derivatives with IC_{50} values in the μM range. The best compound of the series was the tetrazole derivative **5ae** (R_2 = neopentyl) with an IC_{50} of 15.1 μM and 8.12% of residual enzymatic activity at the 100 μM concentration. Regarding the (3*R*) or (3*S*) diastereomeric mixture of 1,5-disubstituted α-amino tetrazole derivatives **5** we observed that the *(3R)* configured stereoisomers were slightly more active with respect to their *(3S)* stereoisomers, in agreement with the results of our computational studies, therefore the subsequent experiments on stereoisomers were performed by using only the *(3R)* diastereomeric mixture of the tetrazole derivatives. In any case, no significative differences in enzymatic activities were observed between racemic compounds and the same tetrazole derivatives with *(3R)* stereochemistry.



Scheme 2. Synthesis of isocyanides. Reagents and conditions: (a) $HCOOC_3H_7$, 90 °C, overnight, quant.; (b) HOBT, DCC, $CHCl_3$, r.t. overnight, 97-91% yields; (c) $POCl_3$, Et_3N, CH_2Cl_2, r.t., 1h, 85−97% yields.



2: R1= Methyl, Isopropyl, ^{t}Butyl, Isobutyl, Neopentyl, Cyclopropyl, Phenyl, 2-Thiophenyl

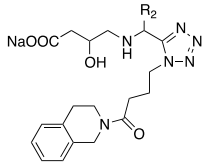
5aa: R1= Methyl
5ab: R1= Isopropyl
5ac: R1= ^{t}Butyl
5ad: R1= Isobutyl
5ae: R1= Neopentyl
5af: R1= Cyclopropyl
5ag: R1= Phenyl
5ah: R1= 2-Thiophenyl
(3*R*)5ae: R1= Neopentyl
(3*R*)5ag: R1= Phenyl
(3*S*)5ag: R1= Phenyl
(3*R*)5ah: R1= 2-Thiophenyl
(3*S*)5ah: R1= 2-Thiophenyl

5ba: R1= Methyl
5bb: R1= Isopropyl
5bc: R1= Neopentyl
5bd: R1= Cyclopropyl
5be: R1= Phenyl
5bf: R1= 2-Thiophenyl
(3*R*)5be: R1= Phenyl
(3*S*)5be: R1= Phenyl
(3*R*)5bf: R1= 2-Thiophenyl
(3*S*)5bf: R1= 2-Thiophenyl

5ca: R1= Isopropyl
5cb: R1= ^{t}Butyl
5cc: R1= Neopentyl
5cd: R1= Cyclopropyl
5ce: R1= 2-Thiophenyl

Scheme 3. Multicomponent Reactions for the synthesis of caspase-1 inhibitors. Reagents and conditions: (a) CH_3OH, Na_2SO_4, Et_3N, r.t., 5 days, 78-20% yields; (b) NaOH, MeOH, r.t., 5 h, 97-50% yields.

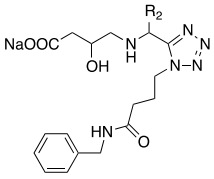
Table 1
In Vitro activity of compounds **5aa-5ah**.[a]..



Compd.	R_2	IC_{50}(μM)	Enzyme activity (% at 100 μM)
5aa	Methyl	ND	IA
5 ab	Isopropyl	ND	77.85
5ac	^{t}Butyl	ND	61.92
5ad	Isobutyl	79.4	43.66
5ae	Neopentyl	15.1	8.12
5af	Cyclopropyl	ND	89.98
5 ag	Phenyl	94.1	47.96
5ah	2-Thiophenyl	ND	77.20
(3*R*)5ae	Neopentyl	12.2	9.71
(3*R*)5 ag	Phenyl	53.5	40.48
(3*S*)5 ag	Phenyl	68.0	48.65
(3*R*)5ah	2-Thiophenyl	32.6	29.00
(3*S*)5ah	2-Thiophenyl	51.4	37.18

[a] IC50 is the concentration of the inhibitor where the enzyme activity is reduced by half (curve fits were performed when the activities at the highest concentration of compounds were less than 60%); ND not determined, and IA inactive.

Table 2
In Vitro activity of compounds **5ba-5bf**.[a]..

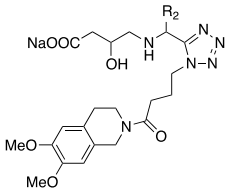


Compd.	R_2	IC_{50}(μM)	Enzyme activity (% at 100 μM)
5ba	Methyl	ND	IA
5bb	Isopropyl	ND	IA
5bc	Neopentyl	ND	59.92
5bd	Cyclopropyl	ND	77.12
5be	Phenyl	ND	IA
5bf	2-Thiophenyl	ND	IA
(3*R*)5be	Phenyl	ND	63.28
(3*S*)5be	Phenyl	ND	62.37
(3*R*)5bf	2-Thiophenyl	77.8	49.65
(3*S*)5bf	2-Thiophenyl	ND	69.80

[a] IC50 is the concentration of the inhibitor where the enzyme activity is reduced by half (curve fits were performed when the activities at the highest concentration of compounds were less than 60%); ND not determined, and IA inactive.

Finally, in order to investigate additional hydrophobic P2' site interactions, two N-alkyl substituents were introduced in the tetrazole derivatives containing tetrahydroisoquinoline and isobutyl or neopentyl residues. Benzyl and 4-fluoro-benzyl R_3 substituents were introduced to give compounds **16aa-16ad** (Scheme 4). A reduction of the IC_{50} value was observed for compounds **16aa-16ab** (R_3 = isobutyl) with respect to their not N-alkylated tetrazole derivates but the same effect was not confirmed for compounds **16ac-16ad** (R_3 = neopentyl) (Table 4). No significative difference in enzymatic activity was observed between compound **16ac** and the same tetrazole derivative with (*3R*) stereochemistry as we found in the previous cases. Taken together, these results on enzymatic activity of diastereomeric mixture support the decision to synthesize

Table 3
In Vitro activity of compounds **5ca-5ce**.[a]..



Compd.	R_2	IC_{50}(μM)	Enzyme activity (% at 100 μM)
5ca	Isopropyl	ND	IA
5 cb	^{t}Butyl	ND	96.73
5cc	Neopentyl	14.6	9.49
5cd	Cyclopropyl	ND	80.56
5ce	2-Thiophenyl	ND	IA

[a] IC50 is the concentration of the inhibitor where the enzyme activity is reduced by half (curve fits were performed when the activities at the highest concentration of compounds were less than 60%); ND not determined, and IA inactive.

and test only the racemic mixture of compound **5** or compound **16** in the subsequent cell-based assays.

2.4. Cytotoxicity and immunomodulatory effect in U937 cells

The 1,5-disubstituted α-amino tetrazole **16aa** and **5ae** were selected for in vitro evaluation of the cytotoxicity and immunomodulatory effect because of their very good caspase-1 inhibition enzymatic activity. The cell line U-937 was used to reproduce in vitro a biological model of inflammation. U-937 is a human cell line expressing many of the monocytic like characteristics. U-937 was differentiated into macrophages with PMA and stimulated with LPS to induce IL-1β production as described in literature [54,55].

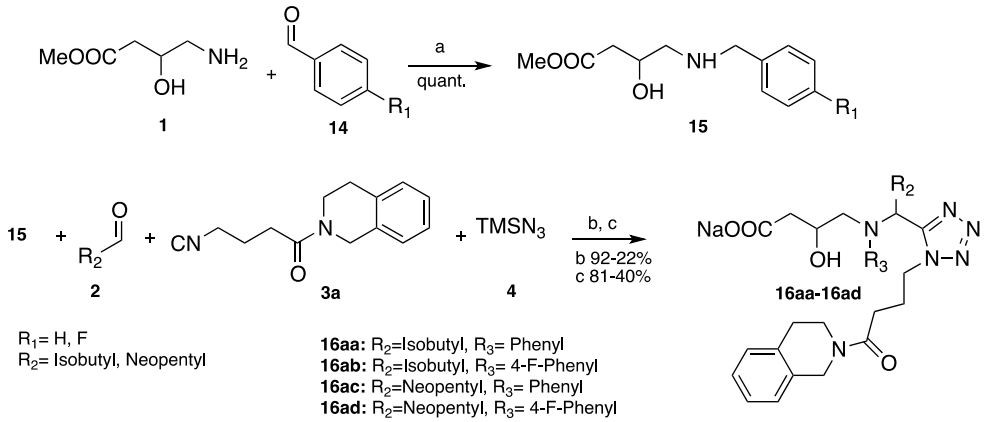
2.4.1. U937 cell growth inhibition

To exclude a cytotoxic effect of **16aa** and **5ae** on human U937 cell line, cells were exposed to increasing concentrations of the synthesized compounds **16aa** and **5ae** ranging from 1 nM to 100 μM for 48h and then evaluated for cell viability and cell growth inhibition by MTT assay. The results illustrated in Fig. 4 show that at the higher concentration the viability for both compounds was reduced by almost 50% while at lowest concentrations the cytotoxic effect is low or is not significant.

In order to evaluate if the toxicity observed at the100 μM concentration was dependent from the solvent used (DMSO) rather than from compound **16aa** or **5ae**, the experiments were repeated by adding DMSO alone at the same concentration used to dilute **16aa** and **5ae**. As showed in Fig. 4, at the concentrations of 100 μM or 10 μM, it can be assumed that the reduction of viability was clearly due to the presence of DMSO rather than to **16aa** or **5ae**.

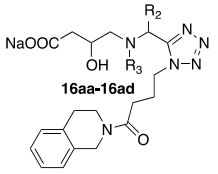
2.4.2. Inhibition of IL-1β release

We initially confirmed that the stimulation with LPS of differentiated U-937 cells induces a release of IL-1β (approximately 200 pg/ml) while unstimulated U-937 cells do not produce significantly IL-1β (data not shown). Subsequently we have studied the capability of compounds **16aa** and **5ae** to inhibit IL-1β release in differentiated U-937 cells stimulated with LPS. The treatment with graded concentrations of **16aa** from 10 μM to 1 nM has been performed simultaneously to LPS (1 μg/ml) treatment for 24h. Alternatively, after LPS stimulation the cells were treated with graded concentrations of **16aa** from 10 μM to 1 nM overnight. After incubation, the supernatants (SN) were collected and stored at −80 °C.



Scheme 4. Synthesis of compounds **16aa-16ad**. Reagents and conditions: (a) i) CH_3OH, r.t., overnight; ii) $NaBH_4$, CH_3OH, r.t., 4.5 h; iii) $SOCl_2$, CH_3OH, r.t. overnight; (b) CH_3OH, Na_2SO_4, Et_3N, r.t., 5 days, 92-22% yields; (c) NaOH, MeOH, r.t., 5 h, 81-40% yields.

Table 4
In Vitro activities of compounds **16aa-16ad**.[a]..



Compd.	R_2	R_3	IC_{50}(μM)	Enzyme activity (% at 100 μM)
16aa	Isobutyl	Benzyl	12.1	2.47
16 ab	Isobutyl	4-F-Benzyl	20.7	2.06
16ac	Neopentyl	Benzyl	10.3	2.91
16ad	Neopentyl	4-F-benzyl	12.7	3.90
***(3R)*16ac**	Neopentyl	Benzyl	15.0	4.45

[a] IC50 is the concentration of the inhibitor where the enzyme activity is reduced by half (curve fits were performed when the activities at the highest concentration of compounds were less than 60%); ND not determined, and IA inactive.

The effect of **16aa** on the IL-1β is showed in Fig. 5. The results are expressed in terms of compound concentration producing 50% inhibition (IC_{50}) of IL-1β release. The results show that **16aa** is able to significantly inhibit the IL-1β production after LPS stimulation at low μM concentrations ($IC_{50} = 0.35$ μM) when used in the presence of LPS (Fig. 5, column A). When the cells were treated with **16aa** after 4h of LPS stimulation the concentration of the compound producing 50% inhibition of IL-1β release increases ($IC_{50} = 0.85$ μM) (Fig. 5, column B).

Our results showed that the secretion of IL-1β was significantly suppressed by **16aa** and that the required concentration is related to the schedule of treatment. We could speculate that during the simultaneous treatment with LPS, **16aa** acts early during the LPS priming. LPS is toll-like receptor (TLR4) ligand, the binding LPS/TLR4 is defined as the priming step, which provides the first signal for NLRP3 inflammasome activation that in turn active Caspase-1 that is involved in the maturation of interleukin IL-1β [56]. After 4 h of pretreatment with LPS, inflammatory machinery is complete and this may require a greater concentration of **16aa** to inhibit Caspase 1. The treatment with graded concentrations of compound

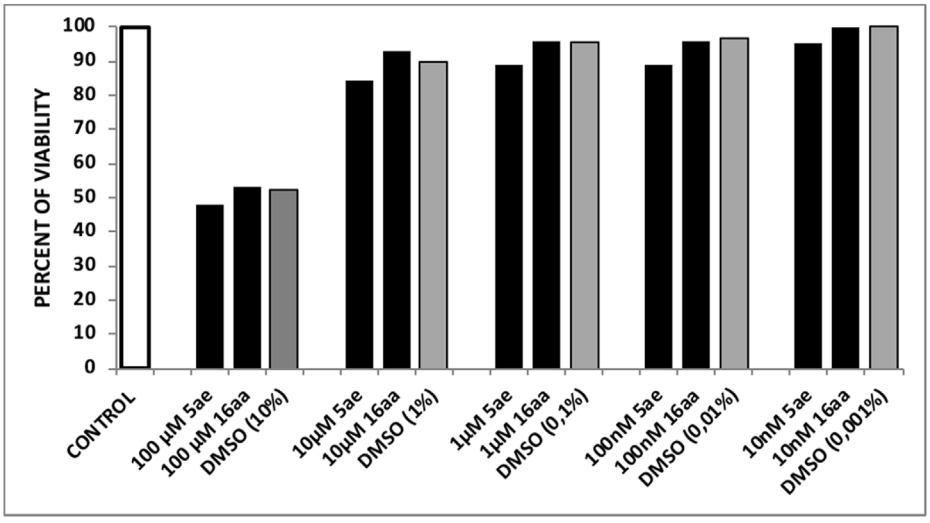


Fig. 4. U937 cell growth inhibition by addition of **16aa** or **5ae**.To evaluate the cell viability after 48 h of treatment with **16aa** or **5ae** has been performed an MTT test. Data are expressed in terms of percentage of cell viability compared to the control group set at 100%. Each percentage value was obtained using the arithmetic average of three independent experiments. The black bars describe the experiments where the compounds **16aa** or **5ae** were added at different concentrations while the grey bars describe the experiments with only the solvent (DMSO) added at the same concentration used to dilute **16aa** or **5ae** in the previous experiments.

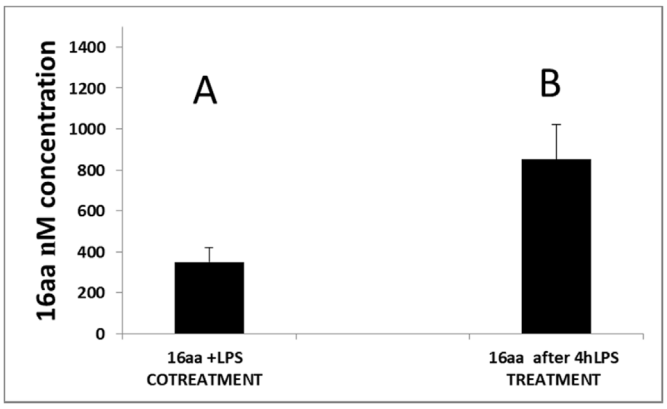


Fig. 5. Inhibition of IL-1β production by 16aa. The results are expressed in terms of compound concentration producing 50% inhibition of IL-1β release (IC_{50}), calculated on the regression line in which the percentage of inhibition were plotted against the logarithm of compound concentration. The IL-1β production was detected by ELISA immunoassay. In the column A are showed the effects of treatment of differentiated U937 with **16aa** simultaneously with LPS. In the column B are showed the effects of treatment of differentiated U937 with **16aa** after 4h from LPS (1 μg/ml) stimulation. Bars represent the fiducial limits of the IC_{50} values.

5ae ranging from 10 μM to 1 nM has been also performed simultaneously to LPS (1 μg/ml) treatment for 24h. The results show that also compound **5ae** is able to significantly inhibit the IL-1β production after LPS stimulation with IC_{50} = 50.06 μM when used in the presence of LPS.

3. Conclusions

Through the rational design and an MCR based synthetic approach of new non-covalent caspase-1 inhibitors we were able to obtain, a noncytotoxic 1,5-disubstituted α-amino tetrazoles able to target the inflammasome caspase-1 pathway that could be of use for the treatment of inflammatory driven diseases. This new class of non-covalent inhibitors was able to inhibit IL-1β release in activated macrophages in the low μM range that is in line with the best activities observed for the known covalent inhibitors that failed in clinical trials, although they showed a much higher enzymatic caspase-1 inhibition activity. The non-covalent mode of action of our inhibitors could be the reason of the greater bioavailability observed because of the lack of an electrophilic substituent in the P1 position. In particular, compound **16aa** could form the basis of further optimization towards novel inflammasome caspase-1 pathway inhibitors, characterized by a good degree of potency and reduced toxicity, suitable for treatment of inflammatory disorders. Further medicinal chemistry and pharmacological studies aimed at increasing caspase-1 inhibition and immunomodulatory effect are in progress.

4. Experimental section

4.1. Chemistry

^1H and ^{13}C NMR spectra were acquired on a Varian 400 MHz, Varian Mercury spectrometer at 400 MHz for ^1H and 100 MHz for ^{13}C. TLC were performed on glass TLC plates, silica gel coated with fluorescent indicator F254 (thickness of 200 μm). Preparative TLC were performed on PTLC glass plates with fluorescent indicator F254 (thickness of 1 mm). Column chromatography was performed using 230–400 mesh silica gel. All chemical reagents and solvents were purchased from commercial sources and used without further purification. CH_2Cl_2 was dried by distillation over CaH_2. The purity of the final compounds was determined by NMR spectroscopy and elemental analysis and was in agreement with the proposed structures with purity ≥95%.

4.2. Synthesis of methyl 4-amino-3-hydroxybutanoate hydrochloride (*1·HCl*)

$SOCl_2$ (190 μL, 2.52 equiv.) was added dropwise at 0 °C to a flask containing CH_3OH (5 mL), then 4-amino-3-hydroxybutanoic acid (250 mg, 2.10 equiv.) was added. The reaction mixture was stirred at r.t. overnight, then the solvent was removed under vacuum. The residue was stirred with hexanes and concentrated under vacuum to give the clean product in a quantitative yield as a thick light-yellow oil. ^1H NMR CD_3OD δ 4.27–4.19 (m, 1H), 3.70 (s, 3H), 3.16–3.09 (m, 1H), 2.95–2.87 (m, 1H) 2.65–2.52 (m, 2H). ^{13}C NMR CD_3OD δ 172.65, 65.55, 52.36, 45.37, 40.46.

4.3. Synthesis of 4-formamidobutanoic acid (*7*)

A solution of 4-aminobutanoic acid **6** (4 g, 38.75 mmol) in propyl formate $HCOOC_3H_7$ (40 mL) and formic acid (2 mL) was refluxed at 90 °C overnight. The solution was concentrated under vacuum and the product was treated with Et_2O to give a white solid in a quantitative yield. m.p. = 104–106 °C. ^1H NMR CD_3OD δ 8.05 (s, 1H), 3.26 (t, *J* = 7.0 Hz, 2H), 2.34 (t, *J* = 7.4 Hz, 2H), 1.85–1.75 (m, 2H). ^{13}C NMR CD_3OD δ 176.94, 163.89, 38.32, 32.22, 25.79.

4.4. General procedure for the synthesis of formamides *11–13*

To a suspension of 4-formamidobutanoic acid **7** (1 equiv) in THF (0.3 M) at r.t., the corresponding amines **8, 9** or **10** (1 equiv.), HOBT (1 equiv.) and DCC (1.1 equiv.) were added. The reaction mixture was stirred at r.t. overnight, then filtered on a celite pad washing with a minimal amount of THF. The solvent was removed under vacuum and the crude product was purified by filtration on a silica gel pad under vacuum eluting first with AcOEt and then with CH_2Cl_2/CH_3OH 9/1 to recover the desired formamides **11–13**.

4.4.1. N-(4-(3,4-dihydroisoquinolin-2(1H)-yl)-4-oxobutyl) formamide (*11*)

Thick light-yellow oil obtained in 97% yield. ^1H NMR CD_3OD δ 8,05 (bs, 1Ha,b), 7.22–7.11 (m, 4Ha,b), 4.68 (s, 2Ha), 4.67 (s, 2Hb), 3.77 (t, *J* = 6 Hz, 2Ha), 3.73 (t, *J* = 6 Hz, 2Hb), 3.32–3.26 (m, 2Ha,b), 2.92 (t, *J* = 6 Hz, 2Hb), 2.84 (t, *J* = 6 Hz, 2Ha), 2.55–2.49 (m, 2Ha,b), 1.90–1.79 (m, 2Ha,b). ^{13}C NMR CD_3OD δ 173.50, 163.79, 136.10, 135.67, 134.39, 134.03, 129.61, 129.36, 127.92, 127.69, 127.53, 127.43, 127.21, 48.19, 45.26, 44.43, 41.20, 38.49, 38.46, 31.67, 31.41, 30.17, 29.30, 25.94, 25.92.

4.4.2. N-(4-(Benzylamino)-4-oxobutyl)formamide (*12*)

White solid obtained in 93% yield. m.p. = 84–86 °C. ^1H NMR CD_3OD δ 8,02 (bs, 1Ha,b), 7.34–7.20 (m, 5Ha,b), 4.36 (s, 2Ha), 4.35 (s, 2Hb), 3.24 (t, *J* = 7 Hz, 2Ha,b), 2.27 (t, *J* = 7.6 Hz, 2Ha,b), 1.87–1.77 (m, 2Ha,b). ^{13}C NMR $CDCl_3$ δ 172.94, 161.98, 138.01, 128.61, 127.66, 127.41, 43.60, 37.64, 33.65, 25.23.

4.4.3. N-(4-(3,4-dihydro-6,7-dimethoxyisoquinolin-2(1H)-yl)-4-oxobutyl)formamide (*13*)

Thick light-yellow oil obtained in 91% yield. ^1H NMR CD_3OD δ 8,09 (bs, 1Ha,b), 6.76 (bs, 2Ha), 6.73 (bs, 2Hb), 4.60 (bs, 2Ha,b), 3.80 (bs, 6Ha,b), 3.75 (t, *J* = 5.8 Hz, 2Ha), 3.69 (t, *J* = 6 Hz, 2Hb), 3.35–3.25 (m, 2Ha,b), 2.82 (t, *J* = 6 Hz, 2Hb), 2.74 (t, *J* = 6 Hz, 2Ha), 2.52 (t, *J* = 6.8 Hz, 2Ha,b), 1.93–1.80 (m, 2Ha,b). ^{13}C NMR CD_3OD δ 173.47, 163.83, 149.39, 149.29, 149.22, 149.17, 128.18, 128.13, 127.68, 126.41, 125.98, 113.03, 112.90, 110.95, 110.78, 56.50, 56.48,

56.44, 47.94, 44.99, 44.55, 41.21, 38.52, 38.50, 31.72, 31.40, 29.72, 28.82, 26.02, 25.98.

4.5. General procedure for the synthesis of isocyanide components **3a-c**

To a solution of formamide **11**–**13** (1 equiv.) in dry CH_2Cl_2 (0.3 M), under nitrogen, Et_3N (5 equiv.) and $POCl_3$ (1.5 equiv.) were added at 0 °C. The reaction mixture was stirred at r.t. for 1 h, then treated with a 20% aqueous solution of Na_2CO_3. The aqueous layer was extracted three times with CH_2Cl_2. The organic phase, dried on Na_2SO_4, was filtered and concentrated under vacuum. The crude product was purified by filtration on a silica gel pad under vacuum, eluting first with CH_2Cl_2 and then with CH_2Cl_2/AcOEt 9/1 to recover the desired products **3a-c**.

4.5.1. 1-(3,4-Dihydroisoquinolin-2(1H)-yl)-4-isocyanobutan-1-one (**3a**)

Light yellow oil obtained in 87% yield. ^{1}H NMR $CDCl_3$ δ 7.25–7.11 (m, 4Ha,b), 4.74 (s, 2Ha), 4.65 (s, 2Hb), 3.84 (t, J = 5.8 Hz, 2Ha), 3.71 (t, J = 5.9 Hz, 2Hb), 3.59–3.54 (m, 2Ha,b), 2.94 (t, J = 5.9 Hz, 2Hb), 2.87 (t, J = 6 Hz, 2Ha), 2.63–2.57 (m, 2Ha,b), 2.12–2.02 (m, 2Ha,b). ^{13}C NMR $CDCl_3$ δ 169.98, 156.52, 156.46, 156.41, 135.08, 134.11, 133.45, 132.34, 128.99, 128.38, 127.12, 126.75, 126.72, 126.55, 126.20, 47.22, 44.35, 43.19, 41.36, 41.30, 41.23, 39.89, 29.59, 29.47, 29.24, 28.57, 24.49, 24.44.

4.5.2. N-benzyl-4-isocyanobutanamide (**3b**)

Thick light-yellow oil obtained in 97% yield. ^{1}H NMR $CDCl_3$ δ 7.38–7.26 (m, 5Ha,b), 5.87 (bs, 1Ha,b), 4.46 (s, 2Ha), 4.44 (s, 2Hb), 3.54–3.49 (m, 2Ha,b), 2.41 (t, J = 7 Hz, 2Hab), 2.09–2.00 (m, 2Ha,b). ^{13}C NMR $CDCl_3$ δ 171.08, 156.34, 138.19, 128.71, 127.69, 127.52, 43.54, 41.08, 41.02, 40.96, 32.16, 24.69.

4.5.3. 1-(6,7-Dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)-4-isocyanobutan-1-one (**3c**)

Light yellow oil obtained in 85% yield. ^{1}H NMR CD_3OD δ 6.77 (s, 2Ha), 6,75 (s, 2Hb), 4.63 (s, 2Ha), 4.61 (s, 2Hb), 3.81 (s, 6Ha), 3.79 (s, 6Hb), 3.79–3.70 (m, 2Ha,b), 3.61–3.53 (m, 2Ha,b), 2.85 (t, J = 5.8 Hz, 2Hb), 2.76 (t, J = 5.8 Hz, 2Ha), 2.63 (t, J = 7.2 Hz, 2Ha,b), 2.06–1.95 (m, 2Ha,b). ^{13}C NMR $CDCl_3$ δ 169.83, 169.78, 156.32, 148.05, 147.94, 147.79, 126.84, 125.72, 125.18, 123.87, 111.59, 111.22, 109.39, 108.91, 55.99, 46.84, 43.94, 43.20, 41.30, 41.24, 41.18, 39.78, 29.52, 29.07, 28.90, 28.01, 24.39, 24.33.

4.6. General procedure for the synthesis of secondary amine hydrochlorides **15a,b·HCl**

To a suspension of 4-amino-3-hydroxybutanoic acid (100 mg, 0.84 mmol) in CH_3OH (3 mL) under nitrogen at r.t., Et_3N (230 μL, 1.68 mmol) was added. After 20 min, benzaldehyde **14a,b** (0.84 mmol) was added and the mixture was stirred at r.t. overnight. The solution was then cooled to 0 °C and $NaBH_4$ (64 mg, 1.68 mmol) was added, and the mixture was stirred for 4.5h at r.t., then concentrated under vacuum to give a thick colorless oil that was dissolved in CH_3OH (5 mL), cooled to 0 °C and treated dropwise with $SOCl_2$ (42 mL, 5.75 mmol) under nitrogen. The reaction mixture was stirred at r.t. overnight then the solvent was removed under vacuum to give the crude product **15a** or **15b** as a solid that was used as such in the following MCRs.

4.6.1. Methyl 4-(benzylamino)-3-hydroxybutanoate hydrochloride (**15a·HCl**)

White solid obtained in quantitative yield. m.p. = 138–140 °C. ^{1}H NMR CD_3OD δ 7.56–7.51 (m, 2H), 7.50–7.44 (m, 3H), 4.38–4.30 (m, 1H), 4.26 (bs, 2H), 3.69 (s, 3H), 3.22–3.16 (m, 1H), 3.06–2.97 (m, 1H), 2.63–2.51 (m, 2H). ^{13}C NMR CD_3OD δ 172.53, 132.28, 131.18, 130.64, 130.23, 64.65, 52.46, 52.30, 52.16, 40.64.

4.6.2. Methyl 4-(4-fluorobenzylamino)-3-hydroxybutanoate hydrochloride (**15b·HCl**)

White solid obtained in quantitative yield. m.p. = 163–165 °C. ^{1}H NMR CD_3OD δ 7.61–7.55 (m, 2H), 7.24–7.16 (m, 2H), 4.38–4.30 (m, 1H), 4.26 (bs, 2H), 3.69 (s, 3H), 3.23–3.17 (m, 1H), 3.06–2.98 (m, 1H), 2.64–2.51 (m, 2H). ^{13}C NMR CD_3OD δ 172.53, 133.67, 133.58, 117.11, 116.89, 64.66, 52.48, 52.31, 51.35, 40.63.

4.7. General MCR procedure for the synthesis of 1,5-disubstituted α-amino tetrazoles **5** and **16**

To a solution of amine hydrochloride component **1** or **15** (1 equiv.) in CH_3OH (0.11 M), Et_3N (1 equiv.), Na_2SO_4, and after 10 min, the oxo component **2** (1 equiv.) were added. The solution was stirred for 15 min and then a solution of isocyanide component **3** (1 equiv.) in CH_3OH (0.44 M) and $TMSN_3$ (1 equiv.) were added. The reaction mixture was stirred at room temperature for 5 days and then filtered on a celite pad to give the crude product that was purified by filtration on a silica gel pad eluting with CH_2Cl_2/Et_2O 9/1, then EtOAc/Acetone 9/1 to give a tick light yellow oil with yields ranging from 20% to 92%. The 4-MCR product was then solved in CH_3OH (0.05 M) was treated with a solution of NaOH 1 M (0.1 M in CH_3OH). The solution was stirred at room temperature for 5h, then the solvent was removed under vacuum and the crude product was purified by filtration on a silica gel pad eluting with EtOAc/Acetone 9/1 and CH_2Cl_2/CH_3OH 8/2 to give the desired product **5** or **16**, with yields ranging from 50% to 96%.

4.7.1. Sodium 4-((1-(1-(4-(3,4-dihydroisoquinolin-2(1H)-yl)-4-oxobutyl)-1H-tetrazol-5-yl)ethyl)amino)-3-hydrxybutanoate (**5aa**)

The title compound was prepared from compound **3a** and **2** (R_1 = methyl) following the general procedure of **5**. Yield MCR 31%, yield hydrolysis 52%, light yellow oil, mixture of atropoisomers. ^{1}H NMR CD_3OD δ 7.20–7.12 (m, 4Ha,b), 4.68 (s, 2Ha), 4.67 (s, 2Hb), 4.64–4.54 (m, 2Ha,b), 4.41–4.31 (m, 1Ha,b) 4.03–3.91 (m, 1Ha,b), 3.76 (t, J = 6 Hz, 2Ha), 3.72 (t, J = 6 Hz, 2Hb), 2.92 (t, J = 6 Hz, 2Hb), 2.84 (t, J = 6 Hz, 2Ha), 2.67–2.52 (m, 1Ha, 2Ha,b), 2.42 (dd, J = 11.2, 7.6 Hz, 1Hb), 2.33–2.18 (m, 4Ha,b), 1.54 (t, J = 1.6 Hz, 3Ha,b), 1.52 (t, J = 1.6 Hz, 3Ha,b). ^{13}C NMR CD_3OD δ 180.08, 172.78, 158.99, 158.90, 136.14, 135.79, 134.43, 134.03, 129.62, 129.41, 127.96, 127.74, 127.56, 127.48, 127.46, 127.32, 69.72, 69.68, 53.63, 53.61, 48.16, 48.05, 48.01, 45.35, 44.45, 43.46, 43.40, 41.34, 30.95, 30.93, 30.65, 30.62, 30.18, 29.38, 26.11, 26.08, 26.04, 24.20, 20.07, 19.71. Anal. Calcd for $C_{20}H_{27}N_6NaO_4$: C, 54.79; H, 6.21; N, 19.17. Found: C, 54.68; H, 6.20; N, 19.14.

4.7.2. Sodium 4-((1-(1-(4-(3,4-dihydroisoquinolin-2(1H)-yl)-4-oxobutyl)-1H-tetrazol-5-yl)-2-methylpropyl)amino)-3-hydroxybutanoate (**5 ab**)

The title compound was prepared from compound **3a** and **2** (R_1 = isopropyl) following the general procedure of **5**. Yield MCR 51%, yield hydrolysis 96%, light yellow oil, mixture of atropoisomers. ^{1}H NMR CD_3OD δ 7.17 (bs, 4Ha,b), 4.68 (s, 2Ha,b), 4.64–4.51 (m, 2Ha,b), 4.01–3.91 (m, 2Ha,b), 3.79 (t, J = 6 Hz, 1Ha), 3.73 (t, J = 6 Hz, 1Hb), 2.93 (t, J = 6 Hz, 1Hb), 2.85 (t, J = 6 Hz, 1Ha), 2.68–2.55 (m, 2Ha,b), 2.50–2.41 (m, 2Ha,b), 2.39–2.22 (m, 4Ha,b), 2.22–2.09 (m, 1Ha,b), 1.07 (d, 6.8 Hz, 3Ha,b), 0.84–0.76 (m, 3Ha,b). ^{13}C NMR CD_3OD δ 179.83, 172.73, 157.93, 157.78, 136.11, 135.76, 134.41, 133.99, 129.63, 129.41, 127.98, 127.76, 127.57, 127.50, 127.46, 127.30, 69.72, 69.36, 60.52, 59.94, 54.08, 53.86, 48.15, 48.03, 47.93, 45.34, 44.43, 43.16, 43.09, 41.33, 33.54, 33.44, 30.92, 30.61, 30.15,

29.37, 26.24, 26.20, 26.16, 19.66, 19.62, 19.56. Anal. Calcd for $C_{22}H_{31}N_6NaO_4$: C, 56.64; H, 6.70; N, 18.01. Found: C, 56.38; H, 6.68; N, 17.96.

4.7.3. Sodium 4-((1-(1-(4-(3,4-dihydroisoquinolin-2(1H)-yl)-4-oxobutyl)-1H-tetrazol-5-yl)-2,2-dimethylpropyl)amino)-3-hydroxybutanoate (**5ac**)

The title compound was prepared starting from compounds **3a** and **2** (R_1 = ^{t}butyl) following the general procedure of **5**. Yield MCR 20%, yield hydrolysis 65%, colorless oil, mixture of atropoisomers. ^{1}H NMR CD_3OD δ 7.20–7.13 (m, 4Ha,b), 4.68 (bs, 2Ha,b), 4.60–4.49 (m, 2Ha,b), 4.00–3.86 (m, 2Ha,b), 3.79 (t, J = 6 Hz, 2Ha), 3.72 (t, J = 6 Hz, 2Hb), 2.93 (t, J = 6 Hz, 2Hb), 2.85 (t, J = 6 Hz, 2Ha), 2.68–2.56 (m, 2Ha,b), 2.48–2.20 (m, 6Ha,b), 0.99 (bs, 9Ha,b).^{13}C NMR CD_3OD δ 172.76, 158.15, 158.01, 136.13, 135.77, 134.44, 134.02, 129.63, 129.41, 127.99, 127.76, 127.57, 127.50, 127.46, 127.31, 69.81, 69.63, 63.16, 62.69, 55.18, 54.99, 48.17, 48.11, 47.98, 45.34, 44.46, 43.29, 42.89, 41.34, 36.95, 36.86, 30.96, 30.64, 30.16, 29.37, 26.94, 26.90, 26.26, 26.22. Anal. Calcd for $C_{23}H_{33}N_6NaO_4$: C, 57.49; H, 6.92; N, 17.49. Found: C, 57.30; H, 6.90; N, 17.45.

4.7.4. Sodium 4-((1-(1-(4-(3,4-dihydroisoquinolin-2(1H)-yl)-4-oxobutyl)-1H-tetrazol-5-yl)-3-methylbutyl)amino)-3-hydroxybutanoate (**5ad**)

The title compound was prepared from compound **3a** and **2** (R_1 = isobutyl) following the general procedure of **5**. Yield MCR 55%, yield hydrolysis 89%, colorless oil, mixture of atropoisomers. ^{1}H NMR CD_3OD δ 7.20–7.14 (m, 4Ha,b), 4.69 (s, 2Ha,b), 4.66–4.57 (m, 2Ha,b), 4.33 (t, J = 7.6 Hz, 1Ha,b), 3.99–3.89 (m, 1Ha,b), 3.78 (t, J = 6 Hz, 2Ha), 3.73 (t, J = 6 Hz, 2Hb), 2.93 (t, J = 6 Hz, 2Hb), 2.85 (t, J = 6 Hz, 2Ha), 2.67–2.59 (m, 2Ha,b), 2.59–2.40 (m, 2Ha,b), 2.39–2.18 (m, 4Ha,b), 1.87–1.69 (m, 2Ha,b), 1.59–1.47 (m, 1Ha,b), 0.95 (d, J = 6.4 Hz, 3Hb), 0.94 (d, J = 6.4 Hz, 3Ha), 0.91 (d, J = 6.8 Hz, 3Ha), 0.90 (d, J = , 3Hb). ^{13}C NMR CD_3OD δ 180.13, 172.70, 172.68, 158.42, 158.40, 158.29, 158.27, 136.13, 135.75, 134.42, 134.00, 129.61, 129.40, 127.96, 127.73, 127.55, 127.48, 127.44, 127.29, 69.83, 69.50, 53.89, 53.62, 52.67, 52.25, 48.15, 48.11, 48.05, 48.04, 45.34, 44.44, 44.25, 44.08, 43.37, 43.32, 41.31, 30.97, 30.96, 30.64, 30.62, 30.18, 29.38, 26.18, 26.14, 26.07, 22.96, 22.90, 22.87, 22.84. Anal. Calcd for $C_{23}H_{33}N_6NaO_4$: C, 57.49; H, 6.92; N, 17.49. Found: C, 57.28; H, 6.89; N, 17.43.

4.7.5. Sodium-4-((1-(1-(4-(3,4-dihydroisoquinolin-2(1H)-yl)-4-oxobutyl)-1H-tetrazol-5-yl)-3,3-dimethylbutyl)amino)-3-hydroxybutanoate (**5ae**)

The title compound was prepared starting from compounds **3a** and **2** (R_1 = neopentyl) following the general procedure of **5**. Yield MCR 78%, hydrolysis 72%, thick light-yellow oil, mixture of atropoisomers. ^{1}H NMR CD_3OD δ 7.21–7.15 (m, 4Ha,b), 4.69 (s, 2Ha,b), 4.66–4.57 (m, 2Ha,b), 4.38–4.33 (m, 1Ha,b), 4.05–3.95 (m, 1Ha,b), 3.78 (t, J = 6 Hz, 2Ha), 3.73 (t, J = 6 Hz, 2Hb), 2.93 (t, J = 6 Hz, 2Hb), 2.85, (t, J = 6Hz, 2Ha), 2.69–2.23 (m, 8Ha,b), 2.00–1.83 (m, 2Ha,b), 0.88 (s, 9Ha,b). ^{13}C NMR CD_3OD δ 172.78, 158.66, 136.15, 135.75, 134.41, 133.97, 129.64, 129.40, 128.00, 127.78, 127.59, 127.48, 127.29, 68.80, 68.56, 53.60, 53.32, 51.42, 51.10, 48.41, 48.27, 48.16, 45.38, 44.47, 41.80, 41.63, 41.36, 31.33, 30.96, 30.63, 30.17, 30.11, 29.38, 26.00. Anal. Calcd for $C_{24}H_{35}N_6NaO_4$: C, 58.29; H, 7.13; N, 16.99. Found: C, 58.18; H, 7.11; N, 16.95.

4.7.6. Sodium (3R)-4-((1-(1-(4-(3,4-dihydroisoquinolin-2(1H)-yl)-4-oxobutyl)-1H-tetrazol-5-yl)-3,3-dimethylbutyl)amino)-3-hydroxybutanoate (**(3R)5ae**)

The title compound was prepared starting from compounds **3a** and **2** (R_1 = neopentyl) and the enantiopure compound **1**(*3R*) following the general procedure of **5**. Yield MCR 60%, hydrolysis 86%, thick light-yellow oil, mixture of atropoisomers. ^{1}H NMR CD_3OD δ 7.21–7.14 (s, 4Ha,b), 4.69 (s, 2Ha,b), 4.65–4.59 (m, 2Ha,b), 4.35–4.29 (m, 1Ha,b), 4.00–3.88 (m, 1Ha,b), 3.79 (t, J = 6.4 Hz, 1Ha), 3.74 (t, J = 5.6 Hz, 1Hb), 2.93 (t, J = 6 Hz, 1Hb), 2.85 (t, J = 6 Hz, 1Ha), 2.69–2.23 (m, 8Ha,b), 1.97–1.81 (m, 2Ha,b), 0.89 (s, 9Ha,b). ^{13}C NMR CD_3OD δ 180.16, 180.09, 172.80, 159.00, 136.17, 135.79, 134.46, 134.04, 129.60, 129.39, 127.97, 127.74, 127.55, 127.45, 127.30, 69.74, 69.50, 54.07, 53.74, 51.65, 51.27, 48.53, 48.21, 45.37, 44.49, 43.36, 43.25, 41.36, 31.33, 31.04, 30.72, 30.20, 30.14, 30.11, 29.91, 29.77, 29.39, 26.03, 26.00. Anal. Calcd for $C_{24}H_{35}N_6NaO_4$: C, 58.29; H, 7.13; N, 16.99. Found: C, 58.20; H, 7.11; N, 16.96.

4.7.7. Sodium-4-((cyclopropyl(1-(4-(3,4-dihydroisoquinolin-2(1H)-yl)-4-oxobutyl)-1H-tetrazol-5-yl)methyl)amino)-3-hydroxybutanoate (**5af**)

The title compound was prepared starting from compounds **3a** and **2** (R_1 = cyclopropyl) following the general procedure of **5**. Yield MCR 38%, hydrolysis 63%, colourless oil, mixture of atropoisomers. ^{1}H NMR CD_3OD δ 7.23–7.12 (m, 4Ha,b), 4.70–4.60 (m, 4Ha,b), 4.04–3.96 (m, 1Ha,b), 3.77 (t, J = 6 Hz, 2Ha), 3.72 (t, J = 6 Hz, 2Hb), 3.64–3.55 (m, 1Ha,b), 2.92 (t, J = 6 Hz, 2Hb), 2.84 (t, J = 6 Hz, 2Ha), 2.72–2.23 (m, 8Ha,b), 1.42–1.28 (m, 1Ha,b), 0.82–0.65 (m, 1Ha,b), 0.57–0.38 (m, 2Ha,b), 0.36–0.22 (m, 1Ha,b). ^{13}C NMR CD_3OD δ 172.73, 157.48, 136.09, 135.73, 134.38, 133.96, 129.62, 129.39, 127.96, 127.74, 127.55, 127.48, 127.45, 127.28, 69.17, 68.75, 59.26, 58.84, 53.86, 53.54, 48.21, 48.13, 45.32, 44.42, 42.81, 42.62, 41.29, 30.95, 30.64, 30.14, 29.35, 26.21, 15.73, 5.75, 2.92. Anal. Calcd for $C_{22}H_{29}N_6NaO_4$: C, 56.89; H, 6.29; N, 18.09. Found: C, 56.66; H, 6.27; N, 18.03.

4.7.8. Sodium-4-(((1-(4-(3,4-dihydroisoquinolin-2(1H)-yl)-4-oxobutyl)-1H-tetrazol-5-yl)(phenyl)methyl)amino)-3-hydroxybutanoate (**5 ag**)

The title compound was prepared starting from compounds **3a** and **2** (R_1 = phenyl) following the general procedure of **5**. Yield MCR 56%, hydrolysis 85%, light yellow oil, mixture of atropoisomers. ^{1}H NMR CD_3OD δ 7.47–7.26 (m, 5Ha,b), 7.21–7.13 (m, 4Ha,b), 5.42 (s, 1Ha,b), 4.65 (bs, 2Ha), 4.55 (bs, 2Hb), 4.49–4.40 (m, 2Ha,b), 4.15–4.05 (m, 1Ha,b), 3.76–3.71 (m, 2Hb), 3.60–3.55 (m, 2Ha), 2.87 (t, J = 6 Hz, 2Ha), 2.83 (t, J = 6.4 Hz, 2Hb), 2.72–2.53 (m, 2Ha,b), 2.45–2.30 (m, 4Ha,b), 2.09–1.98 (m, 2Ha,b). ^{13}C NMR CD_3OD δ 180.00, 172.58, 157.75, 139.40, 139.23, 136.15, 135.75, 134.40, 133.95, 130.10, 129.64, 129.37, 128.95, 127.97, 127.74, 127.55, 127.45, 127.30, 69.62, 69.47, 58.37, 58.16, 54.19, 53.95, 48.09, 47.99, 45.30, 44.37, 43.39, 43.38, 41.28, 30.73, 30.43, 30.14, 29.36, 25.70. Anal. Calcd for $C_{25}H_{29}N_6NaO_4$: C, 59.99; H, 5.84; N, 16.79. Found: C, 59.73; H, 5.82; N, 16.77.

4.7.9. Sodium (3R)-4-(((1-(4-(3,4-dihydroisoquinolin-2(1H)-yl)-4-oxobutyl)-1H-tetrazol-5-yl)(phenyl)methyl)amino)-3-hydroxybutanoate (**(3R)5 ag**)

The title compound was prepared starting from compounds **3a** and **2** (R_1 = phenyl) and the enantiopure compound **1**(*3R*) following the general procedure of **5**. Yield MCR 57%, hydrolysis 63%, thick light-yellow oil, mixture of atropoisomers. ^{1}H NMR CD_3OD δ 7.47–7.26 (m, 5Ha,b), 7.21–7.13 (m, 4Ha,b), 5.42 (s, 1Ha,b), 4.65 (bs, 2Ha), 4.55 (bs, 2Hb), 4.49–4.40 (m, 2Ha,b), 4.15–4.05 (m, 1Ha,b), 3.76–3.71 (m, 2Hb), 3.60–3.55 (m, 2Ha), 2.87 (t, J = 6 Hz, 2Ha), 2.83 (t, J = 6.4 Hz, 2Hb), 2.72–2.53 (m, 2Ha,b), 2.45–2.30 (m, 4Ha,b), 2.09–1.98 (m, 2Ha,b). ^{13}C NMR CD_3OD δ 179.92, 172.60, 157.75, 139.42, 139.24, 136.16, 135.76, 134.41, 133.97, 130.11, 129.63, 129.40, 128.98, 127.98, 127.75, 127.57, 127.47, 127.31, 69.61, 69.46, 58.35, 58.12, 54.14, 53.92, 48.10, 48.01, 45.32, 44.39, 43.33, 41.30, 30.73, 30.43, 30.15, 29.37, 25.72. Anal. Calcd for $C_{25}H_{29}N_6NaO_4$: C, 59.99; H, 5.84; N, 16.79. Found: C, 59.78; H, 5.83; N, 16.77.

*4.7.10. Sodium (3S)-4-(((1-(4-(3,4-dihydroisoquinolin-2(1H)-yl)-4-oxobutyl)-1H-tetrazol-5-yl)(phenyl)methyl)amino)-3-hydroxybutanoate (**(3S)5 ag**)*

The title compound was prepared starting from compounds **3a** and **2** (R_1 = phenyl) and the enantiopure compound **1**(*3S*) following the general procedure of **5**. Yield MCR 45%, hydrolysis 80%, thick light-yellow oil, mixture of atropoisomers. ^{1}H NMR CD_3OD δ 7.47–7.26 (m, 5Ha,b), 7.21–7.13 (m, 4Ha,b), 5.42 (s, 1Ha,b), 4.65 (bs, 2Ha), 4.55 (bs, 2Hb), 4.49–4.40 (m, 2Ha,b), 4.15–4.05 (m, 1Ha,b), 3.76–3.71 (m, 2Hb), 3.60–3.55 (m, 2Ha), 2.87 (t, J = 6 Hz, 2Ha), 2.83 (t, J = 6.4 Hz, 2Hb), 2.72–2.53 (m, 2Ha,b), 2.45–2.30 (m, 4Ha,b), 2.09–1.98 (m, 2Ha,b). ^{13}C NMR CD_3OD δ 180.15, 180.10, 172.57, 157.79, 157.76, 139.40, 139.24, 136.15, 135.74, 134.41, 133.94, 130.11, 129.63, 129.38, 128.96, 127.97, 127.75, 127.56, 127.44, 127.28, 69.62, 69.47, 58.39, 58.18, 54.22, 53.97, 48.09, 47.99, 45.30, 44.37, 43.39, 43.34, 41.28, 30.73, 30.43, 30.14, 29.35, 25.68. Anal. Calcd for $C_{25}H_{29}N_6NaO_4$: C, 59.99; H, 5.84; N, 16.79. Found: C, 59.81; H, 5.83; N, 16.78.

*4.7.11. Sodium 4-(((1-(4-(3,4-dihydroisoquinolin-2(1H)-yl)-4-oxobutyl)-1H-tetrazol-5-yl)(thiophen-2-yl)methyl)amino)-3-hydroxybutanoate (**5ah**)*

The title compound was prepared starting from compounds **3a** and **2** (R_1 = 2-thiophenyl) following the general procedure of **5**. Yield MCR 26%, hydrolysis 69%, thick light-yellow oil, mixture of atropoisomers. ^{1}H NMR CD_3OD δ 7.39–7.34 (m, 1Ha,b), 7.20–7.10 (m, 5Ha,b), 6.98–6.92 (m, 1Ha,b), 5.77 (s, 1Ha,b), 4.65 (s, 2Ha), 4.58 (s, 2Hb), 4.56–4.47 (m, 2Ha,b), 4.13–4.02 (m, 1Ha,b), 3.74 (t, J = 6 Hz, 2Hb), 3.61 (t, J = 6 Hz, 2Ha), 2.87 (t, J = 6 Hz, 2Ha), 2.82 (t, J = 6 Hz, 2Hb), 2.75–2.54 (m, 2Ha,b), 2.50–2.28 (m, 4Ha,b), 2.17–2.05 (m, 2Ha,b). ^{13}C NMR CD_3OD δ 178.50, 172.60, 157.38, 142.75, 142.56, 136.14, 135.76, 134.40, 133.95, 129.62, 129.40, 128.12, 127.96, 127.75, 127.66, 127.57, 127.47, 127.31, 69.29, 69.08, 53.83, 53.72, 53.61, 53.56, 48.16, 48.08, 45.31, 44.38, 42.57, 41.28, 30.75, 30.43, 30.13, 29.36, 25.79. Anal. Calcd for $C_{23}H_{27}N_6NaO_4S$: C, 54.53; H, 5.37; N, 16.59. Found: C, 54.41; H, 5.36; N, 16.56.

*4.7.12. Sodium (3R)-4-(((1-(4-(3,4-dihydroisoquinolin-2(1H)-yl)-4-oxobutyl)-1H-tetrazol-5-yl)(thiophen-2-yl)methyl)amino)-3-hydroxybutanoate (**(3R)5ah**)*

The title compound was prepared starting from compounds **3a** and **2** (R_1 = 2-thiophenyl) and the enantiopure compound **1**(*3R*) following the general procedure of **5**. Yield MCR 31%, hydrolysis 57%, thick light-yellow oil, mixture of atropoisomers. ^{1}H NMR CD_3OD δ 7.39–7.34 (m, 1Ha,b), 7.21–7.10 (m, 5Ha,b), 6.98–6.94 (m, 1Ha,b), 5.78 (s, 1Ha), 5.77 (s, 1Hb), 4.66 (s, 2Ha), 4.59 (s, 2Hb), 4.57–4.52 (m, 2Ha,b), 4.10–4.02 (m, 1Ha,b), 3.75 (t, J = 5.6 Hz, 2Hb), 3.62 (t, J = 6.4 Hz, 2Ha), 2.88 (t, J = 6 Hz, 2Ha), 2.83 (t, J = 6 Hz, 2Hb), 2.74–2.54 (m, 2Ha,b), 2.49–2.42 (m, 2Ha,b), 2.33–2.29 (m, 2Ha,b), 2.16–2.07 (m, 2Ha,b). ^{13}C NMR CD_3OD δ 178.66, 172.63, 157.41, 142.82, 142.63, 136.16, 135.78, 134.42, 133.97, 129.62, 129.39, 128.07, 127.96, 127.75, 127.57, 127.47, 127.32, 69.40, 69.18, 53.88, 53.78, 53.67, 53.63, 48.18, 48.11, 45.33, 44.41, 42.71, 41.31, 30.77, 30.45, 30.16, 29.37, 25.79. Anal. Calcd for $C_{23}H_{27}N_6NaO_4S$: C, 54.53; H, 5.37; N, 16.59. Found: C, 54.39; H, 5.35; N, 16.54.

*4.7.13. Sodium (3S)-4-(((1-(4-(3,4-dihydroisoquinolin-2(1H)-yl)-4-oxobutyl)-1H-tetrazol-5-yl)(thiophen-2-yl)methyl)amino)-3-hydroxybutanoate (**(3S)5ah**)*

The title compound was prepared starting from compounds **3a** and **2** (R_1 = 2-thiophenyl) and the enantiopure compound **1**(*3S*) following the general procedure of **5**. Yield MCR 27%, hydrolysis 69%, thick light-yellow oil, mixture of atropoisomers. ^{1}H NMR CD_3OD δ 7.39–7.35 (m, 1Ha,b), 7.21–7.11 (m, 5Ha,b), 6.98–6.94 (m, 1Ha,b), 5.78 (s, 1Ha), 5.77 (s, 1Hb), 4.65 (bs, 2Ha), 4.58 (bs, 2Hb), 4.57–4.51 (m, 2Ha,b), 4.10–4.02 (m, 1Ha,b), 3.75 (t, J = 5.6 Hz, 2Hb), 3.62 (t, J = 5.6 Hz, 2Ha), 2.88 (t, J = 6 Hz, 2Ha), 2.83 (t, J = 6 Hz, 2Hb), 2.74–2.54 (m, 2Ha,b), 2.49–2.42 (m, 2Ha,b), 2.34–2.27 (m, 2Ha,b), 2.16–2.08 (m, 2Ha,b). ^{13}C NMR CD_3OD δ 179.93, 172.67, 157.47, 142.95, 142.73, 136.19, 135.80, 134.46, 134.01, 129.61, 129.38, 127.98, 127.93, 127.84, 127.75, 127.57, 127.47, 127.32, 69.74, 69.49, 53.97, 53.90, 53.75, 48.23, 48.15, 45.36, 44.45, 43.29, 41.34, 30.82, 30.52, 30.18, 29.38, 25.80. Anal. Calcd for $C_{23}H_{27}N_6NaO_4S$: C, 54.53; H, 5.37; N, 16.59. Found: C, 54.44; H, 5.36; N, 16.57.

*4.7.14. Sodium 4-((1-(1-(4-(benzylamino)-4-oxobutyl)-1H-tetrazol-5-yl)ethyl)amino)-3-hydroxybutanoate (**5ba**)*

The title compound was prepared starting from compounds **3b** and **2** (R_1 = methyl) following the general procedure of **5**. Yield MCR 55%, hydrolysis 65%, light-yellow oil, mixture of atropoisomers. ^{1}H NMR CD_3OD δ 7.38–7.19 (m, 5Ha,b), 4.60–4.51 (m, 2Ha,b), 4.38–4.29 (m, 1Ha,b), 4.36 (s, 2Ha,b), 4.03–3.93 (m, 1Ha,b), 2.63 (dd, J = 12, 3.6 Hz, 1Ha), 2.58–2.55 (m, 1Ha,b), 2.43 (dd, J = 12, 7.6 Hz, 1Hb), 2.39–2.21 (m, 6Ha,b), 1.54 (d, J = 2 Hz, 3Ha), 1.52 (d, J = 2 Hz, 3Hb). ^{13}C NMR CD_3OD δ 179.88, 174.26, 174.24, 158.95, 158.85, 140.03, 129.59, 128.69, 128.24, 69.71, 69.61, 53.68, 53.54, 48.07, 48.00, 44.18, 43.45, 43.35, 33.38, 33.33, 26.68, 26.66, 20.04, 19.72. Anal. Calcd for $C_{18}H_{25}N_6NaO_4$: C, 52.42; H, 6.11; N, 20.38. Found: C, 52.26; H, 6.09; N, 20.33.

*4.7.15. Sodium 4-((1-(1-(4-(benzylamino)-4-oxobutyl)-1H-tetrazol-5-yl)-2-methylpropyl)amino)-3-hydroxybutanoate (**5bb**)*

The title compound was prepared starting from compounds **3b** and **2** (R_1 = isopropyl) following the general procedure of **5**. Yield MCR 65%, hydrolysis 58%, thick colorless oil, mixture of atropoisomers. ^{1}H NMR CD_3OD δ 7.34–7.21 (m, 5Ha,b), 4.59–4.49 (m, 2Ha,b), 4.37 (s, 2Ha,b), 3.99–3.87 (m, 2Ha,b), 2.49–2.12 (m, 9Ha,b), 1.07 (d, 6.8 Hz, 3Ha), 1.06 (d, 6.8 Hz, 3Hb), 0.82 (d, 6.8 Hz, 3Hb), 0.80 (d, 6.8 Hz, 3Ha). ^{13}C NMR CD_3OD δ 180.12, 174.21, 157.95, 157.81, 140.04, 129.55, 128.62, 128.20, 69.91, 69.49, 60.80, 60.14, 54.30, 53.94, 48.09, 47.97, 44.17, 43.40, 43.35, 33.55, 33.46, 33.37, 33.31, 26.82, 26.73, 26.69, 19.69, 19.58, 19.53. Anal. Calcd for $C_{20}H_{29}N_6NaO_4$: C, 54.54; H, 6.64; N, 19.08. Found: C, 54.33; H, 6.62; N, 19.04.

*4.7.16. Sodium 4-((1-(1-(4-(benzylamino)-4-oxobutyl)-1H-tetrazol-5-yl)-3,3-dimethylbutyl)amino)-3-hydroxybutanoate (**5bc**)*

The title compound was prepared starting from compounds **3b** and **2** (R_1 = neopentyl) following the general procedure of **5**. Yield MCR 78%, hydrolysis 60%, light-yellow oil, mixture of atropoisomers. ^{1}H NMR CD_3OD δ 7.34–7.27 (m, 4Ha,b), 7.26–7.21 (m,1Ha,b), 4.64–4.52 (m, 2Ha,b), 4.37 (s, 2Ha,b), 4.35–4.29 (m, 1H), 4.05–3.93 (m, 1Ha,b), 2.60–2.50 (m, 1Ha,b), 2.45–2.26 (m, 7Ha,b), 1.96–1.89 (m, 1Ha,b), 1.86–1.80 (m, 1Ha,b), 0.89 (s, 9Ha), 0.88 (s, 9Hb). ^{13}C NMR CD_3OD δ 177.83, 174.78, 174.18, 158.73, 139.96,129.55, 128.61, 128.20, 69.10, 68.81, 53.79, 53.43, 51.61, 51.20, 48.52, 48.40, 48.15, 48.10, 44.16, 42.31, 42.10, 33.34, 31.32, 30.27, 30.17, 30.12, 30.10, 26.57. Anal. Calcd for $C_{22}H_{33}N_6NaO_4$: C, 56.40; H, 7.10; N, 17.94. Found: C, 56.22; H, 7.07; N, 17.90.

*4.7.17. Sodium 4-(((1-(4-(benzylamino)-4-oxobutyl)-1H-tetrazol-5-yl)(cyclopropyl)methyl)amino)-3-hydroxybutanoate (**5bd**)*

The title compound was prepared starting from compounds **3b** and **2** (R_1 = cyclopropyl) following the general procedure of **5**. Yield MCR 54%, hydrolysis 65%, colourless oil, mixture of atropoisomers. ^{1}H NMR CD_3OD δ 7.37–7.18 (m, 5Ha,b), 4.65–4.56 (m, 2Ha,b), 4.36 (bs, 3Ha,b), 4.07–3.93 (m, 1Ha,b), 2.72–2.60 (m, 1Ha,b), 2.57–2.44 (m, 1Ha,b), 2.43–2.16 (m, 6Ha,b), 1.41–1.24 (m, 1Ha,b), 0.79–0.65 (m, 1Ha,b), 0.57–0.41 (m, 2Ha,b), 0.35–0.22 (m, 1Ha,b). ^{13}C NMR CD_3OD δ 180.09, 174.25, 173.97, 157.76, 140.00, 129.54, 128.63,

128.61, 128.20, 69.67, 69.21, 59.65, 59.13, 54.18, 54.12, 53.93, 48.26, 48.23, 48.05, 48.01, 44.19, 41.57, 41.50, 33.44, 33.42, 33.25, 26.80, 26.77, 26.68, 26.39, 15.86, 5.69, 5.60, 3.04, 2.91. Anal. Calcd for $C_{20}H_{27}N_6NaO_4$: C, 54.79; H, 6.21; N, 19.17. Found: C, 54.65; H, 6.19; N, 19.13.

4.7.18. Sodium 4-(((1-(4-(benzylamino)-4-oxobutyl)-1H-tetrazol-5-yl)(phenyl)methyl)amino)-3-hydroxybutanoate (5be)

The title compound was prepared starting from compounds **3b** and **2** (R_1 = phenyl) following the general procedure of **5**. Yield MCR 51%, hydrolysis 55%, light yellow oil, mixture of atropoisomers. ^{1}H NMR CD_3OD δ 7.44–7.20 (m, 10Ha,b), 5.38 (s, 1Ha,b), 4.40 (q, J = 8 Hz, 2Ha,b), 4.34 (s, 2Ha,b), 4.13–4.03 (m, 1Ha,b), 2.69–2.51 (m, 2Ha,b), 2.39–2.26 (m, 2Ha,b), 2.25–2.17 (m, 2Ha,b), 2.01–1.90 (m, 2Ha,b).^{13}C NMR CD_3OD δ 174.05, 157.69, 139.97, 139.26, 139.11, 130.15, 129.69, 129.56, 128.95, 128.61, 128.22, 69.55, 69.42, 58.41, 58.24, 54.09, 53.94, 47.99, 44.11, 43.33, 43.24, 33.18, 26.31, 26.29. Anal. Calcd for $C_{23}H_{27}N_6NaO_4$: C, 58.22; H, 5.74; N, 17.71. Found: C, 58.13; H, 5.72; N, 17.68.

*4.7.19. Sodium (3R)-4-(((1-(4-(benzylamino)-4-oxobutyl)-1H-tetrazol-5-yl)(phenyl)methyl)amino)-3-hydroxybutanoate (**(3R)5be**)*

The title compound was prepared starting from compounds **3b** and **2** (R_1 = phenyl) and the enantiopure compound **1**(*3R*) following the general procedure of **5**. Yield MCR 52%, hydrolysis 54%, light-yellow oil, mixture of atropoisomers. ^{1}H NMR CD_3OD δ 7.44–7.22 (m, 10Ha,b), 5.38 (s, 1Ha), 5.37 (s, 1Hb), 4.41 (q, J = 8 Hz, 2Ha,b), 4.34 (s, 2Ha,b), 4.11–4.03 (m, 1Ha,b), 2.69–2.52 (m, 2Ha,b), 2.36–2.26 (m, 2Ha,b), 2.25–2.22 (m, 2Ha,b), 2.01–1.88 (m, 2Ha,b). ^{13}C NMR CD_3OD δ 180.13, 180.06, 174.03, 174.02, 157.69, 157.66, 139.98, 139.30, 139.14, 130.11, 129.64, 129.54, 128.92, 128.90, 128.61, 128.20, 69.60, 69.50, 58.42, 58.25, 54.16, 53.98, 47.99, 44.11, 43.49, 43.40, 33.19, 26.29. Anal. Calcd for $C_{23}H_{27}N_6NaO_4$: C, 58.22; H, 5.74; N, 17.71. Found: C, 58.03; H, 5.72; N, 17.67.

*4.7.20. Sodium (3S)-4-(((1-(4-(benzylamino)-4-oxobutyl)-1H-tetrazol-5-yl)(phenyl)methyl)amino)-3-hydroxybutanoate (**(3S)5be**)*

The title compound was prepared starting from compounds **3b** and **2** (R_1 = phenyl) and the enantiopure compound **1**(*3S*) following the general procedure of **5**. Yield MCR 45%, hydrolysis 64%, light-yellow oil, mixture of atropoisomers. ^{1}H NMR CD_3OD δ 7.44–7.22 (m, 10Ha,b), 5.38 (s, 1Ha), 5.37 (s, 1Hb), 4.42 (q, J = 8 Hz, 2Ha,b), 4.34 (s, 2Ha,b), 4.11–4.03 (m, 1Ha,b), 2.69–2.52 (m, 2Ha,b), 2.32–2.28 (m, 2Ha,b), 2.25–2.20 (m, 2Ha,b), 2.03–1.94 (m, 2Ha,b). ^{13}C NMR CD_3OD δ 180.13, 180.06, 174.03, 157.69, 139.97, 139.31, 139.16, 130.11, 129.63, 129.54, 128.90, 128.60, 128.19, 69.61, 69.51, 58.43, 58.27, 54.15, 54.00, 48.00, 44.13, 43.44, 43.36, 33.21, 26.29. Anal. Calcd for $C_{23}H_{27}N_6NaO_4$: C, 58.22; H, 5.74; N, 17.71. Found: C, 58.08; H, 5.71; N, 17.69.

*4.7.21. Sodium 4-(((1-(4-(benzylamino)-4-oxobutyl)-1H-tetrazol-5-yl)(thiophen-2-yl)methyl)amino)-3-hydroxybutanoate (**5bf**)*

The title compound was prepared starting from compounds **3b** and **2** (R_1 = 2-tiophenyl) following the general procedure of **5**. Yield MCR 43%, hydrolysis 97%, light-yellow oil, mixture of atropoisomers. ^{1}H NMR CD_3OD δ 7.42–7.39 (m 1Ha,b), 7.34–7.21 (m, 5Ha,b), 7.10 (dd, J = 10.8, 3.2 Hz, 1Ha,b), 7.00–6.96 (m, 1Ha,b), 5.74 (s, 1Ha), 5,73 (s, 1Hb), 4.51–4.45 (m, 2Ha,b), 4.35 (s, 2Ha,b), 4.11–4.03 (m, 1Ha,b), 2.72 (dd, J = 12, 3.6 Hz, 1Hb), 2.66–2.63 (m, 1Ha,b), 2.58 (dd, J = 12, 7.6 Hz, 1Ha), 2.42–2.24 (m, 4Ha,b), 2.11–2.02 (m, 2Ha,b). ^{13}C NMR CD_3OD δ 180.11, 174.11, 157.32, 142.71, 142.54, 139.97, 129.55, 128.63, 128.21, 128.01, 127.98, 127.97, 127.87, 127.58, 127.54, 69.51, 69.32, 53.98, 53.83, 53.78, 53.70, 48.21, 44.15, 42.99, 33.25, 26.40. Anal. Calcd for $C_{21}H_{25}N_6NaO_4S$: C, 52.49; H, 5.24; N, 17.49. Found: C, 52.34; H, 5.22; N, 17.46.

*4.7.22. Sodium (3R)-4-(((1-(4-(benzylamino)-4-oxobutyl)-1H-tetrazol-5-yl)(thiophen-2-yl)methyl)amino)-3-hydroxybutanoate (**(3R)5bf**)*

The title compound was prepared starting from compounds **3b** and **2** (R_1 = 2-tiophenyl) and the enantiopure compound **1**(*3R*) following the general procedure of **5**. Yield MCR 39%, hydrolysis 55%, light-yellow oil, mixture of atropoisomers. ^{1}H NMR CD_3OD δ 7.42–7.40 (m, 1Ha), 7.40–7.38 (m, 1Hb), 7.34–7.21 (m, 5Ha,b), 7.13–7.11 (m, 1Ha), 7.11–7.08 (m, 1Hb), 6.99 (dd, J = 4.8, 2.4 Hz, 1Ha), 6.97 (dd, J = 5.2, 2.4 Hz, 1Hb), 5.74 (s, 1Ha), 5.73 (s, 1Hb), 4.52–4.45 (m, 2Ha,b), 4.35 (s, 2Ha,b), 4.10–4.01 (m, 1Ha,b), 2.71 (dd, J = 12, 3.6 Hz, 1Ha), 2.65–2.62 (m, 1Ha,b), 2.56 (dd, J = 12, 7.6 Hz, 1Hb), 2.35–2.23 (m, 4Ha,b), 2.12–2.02 (m, 2Ha,b). ^{13}C NMR CD_3OD δ 180.13, 180.07, 174.10, 157.39, 157.36, 142.81, 142.63, 140.00, 129.54, 128.63, 128.20, 127.96, 127.95, 127.93, 127.80, 127.49, 127.46, 69.72, 69.53, 54.04, 53.89, 53.81, 53.75, 48.24, 44.16, 43.39, 43.34, 33.30, 26.41. Anal. Calcd for $C_{21}H_{25}N_6NaO_4S$: C, 52.49; H, 5.24; N, 17.49. Found: C, 52.31; H, 5.22; N, 17.44.

*4.7.23. Sodium (3S)-4-(((1-(4-(benzylamino)-4-oxobutyl)-1H-tetrazol-5-yl)(thiophen-2-yl)methyl)amino)-3-hydroxybutanoate (**(3S)5bf**)*

The title compound was prepared starting from compounds **3b** and **2** (R_1 = 2-tiophenyl) and the enantiopure compound **1**(*3S*) following the general procedure of **5**. Yield MCR 39%, hydrolysis 88%, light-yellow oil, mixture of atropoisomers. ^{1}H NMR CD_3OD δ 7.40 (dd, J = 2, 1.2 Hz, 1Ha), 7.39 (dd, J = 2, 1.2 Hz, 1Hb), 7.34–7.21 (m, 5Ha,b), 7.13–7.11 (m, 1Ha), 7.11–7.08 (m, 1Hb), 6.99 (dd, J = 3.6, 2.8 Hz, 1Ha), 6.97 (dd, J = 3.6, 2.8 Hz, 1Hb), 5.74 (s, 1Ha), 5.73 (s, 1Hb), 4.53–4.47 (m, 2Ha,b), 4.35 (s, 2Ha,b), 4.09–4.01 (m, 1Ha,b), 2.71(dd, J = 12, 4 Hz, 1Ha), 2.65–2.62 (m, 1Ha,b), 2.56 (dd, J = 12.4, 7.6 Hz, 1Hb), 2.34–2.25 (m, 4Ha,b), 2.12–2.04 (m, 2Ha,b). ^{13}C NMR CD_3OD δ 180.15, 180.10, 174.09, 157.38, 157.36, 142.77, 142.59, 139.99, 129.54, 128.62, 128.19, 127.97, 127.96, 127.82, 127.52, 127.48, 69.70, 69.50, 54.03, 53.90, 53.87, 53.77, 48.23, 44.15, 43.38, 43.34, 33.28, 26.39. Anal. Calcd for $C_{21}H_{25}N_6NaO_4S$: C, 52.49; H, 5.24; N, 17.49. Found: C, 52.38; H, 5.21; N, 17.47.

*4.7.24. Sodium 4-((1-(1-(4-(6,7-dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)-4-oxobutyl)-1H-tetrazol-5-yl)-2-methylpropyl)amino)-3-hydroxybutanoate (**5ca**)*

The title compound was prepared starting from compounds **3c** and **2** (R_1 = isopropyl) following the general procedure of **5**. Yield MCR 72%, hydrolysis 67%, colorless oil, mixture of atropoisomers. ^{1}H NMR CD_3OD δ 6.83–6.71 (m, 2Ha,b), 4.62 (s, 2Ha), 4.60 (s, 2Hb), 4.60–4.50 (m, 2Ha,b), 4.03–3.95 (m, 2Ha,b), 3.81 (bs, 6Ha), 3.80 (bs, 6Hb), 3.77 (t, J = 6 Hz, 2Ha), 3.70 (t, J = 6.4 Hz, 2Hb), 2.84 (t, J = 5.6 Hz, 2Hb), 2.77 (t, J = 6 Hz, 2Ha), 2.66–2.56 (m, 2Ha,b), 2.52–2.45 (m, 2Ha,b), 2.44–2.11 (m, 5Ha,b), 1.07 (d, 6.8 Hz, 3Ha,b), 0.86–0.79 (m, 3Ha,b). ^{13}C NMR CD_3OD δ 172.65, 172.60, 172.50, 172.45, 157.55, 157.49, 149.46, 149.34, 149.30, 149.23, 128.20, 127.75, 126.43, 125.92, 113.10, 112.98, 111.04, 110.86, 68.76, 68.42, 60.22, 60.19, 59.77, 59.74, 56.54, 56.50, 56.47, 53.63, 53.50, 47.99, 47.93, 47.86, 45.06, 44.50, 44.45, 41.50, 41.42, 41.31, 33.43, 33.35, 30.89, 30.52, 29.65, 28.84, 26.21, 26.19, 19.53, 19.49, 19.47. Anal. Calcd for $C_{24}H_{35}N_6NaO_6$: C, 54.74; H, 6.70; N, 15.96. Found: C, 54.57; H, 6.67; N, 15.92.

*4.7.25. Sodium 4-((1-(1-(4-(6,7-dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)-4-oxobutyl)-1H-tetrazol-5-yl)-2,2-dimethylpropyl)amino)-3-hydroxybutanoate (**5 cb**)*

The title compound was prepared starting from compounds **3c**

and **2** (R_1 = ^{t}butyl) following the general procedure of **5**. Yield MCR 20%, hydrolysis 55%, light-yellow oil, mixture of atropoisomers. ^{1}H NMR CD_3OD δ 6.78–6.73 (m, 2Ha,b), 4.62 (bs, 2Ha), 4.61 (bs, 2Hb), 4.60–4.49 (m, 2Ha,b), 4.05–3.95 (m, 1Ha,b), 3.93 (s,1Ha), 3.91 (s,1Hb), 3.81 (s, 6Ha), 3.80 (s, 6Hb), 3.78 (t, *J* = 6 Hz, 2Ha), 3.71 (t, *J* = 6 Hz, 2Hb), 2.85 (t, *J* = 5.6 Hz, 2Ha), 2.77 (t, *J* = 6.4 Hz, 2Hb), 2.66–2.58 (m, 2Ha,b), 2.48–2.25 (m, 6Ha,b), 1.00 (bs, 9Ha), 0.99 (bs, 9Hb). ^{13}C NMR CD_3OD δ 172.72, 172.65, 157.95, 149.42, 149.31, 149.26, 149.21, 128.19, 127.77, 126.44, 125.94, 113.03, 112.91, 110.98, 110.80, 69.39, 69.22, 63.14, 56.56, 56.53, 56.48, 56.46, 54.87, 48.10, 47.91, 45.09, 44.55, 41.35, 37.00, 30.96, 30.59, 29.70, 28.88, 27.69, 26.94, 26.91, 26.36. Anal. Calcd for $C_{25}H_{37}N_6NaO_6$: C, 55.54; H, 6.90; N, 15.55. Found: C, 55.34; H, 6.88; N, 15.52.

*4.7.26. Sodium 4-((1-(1-(4-(6,7-dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)-4-oxobutyl)-1H-tetrazol-5-yl)-3,3-dimethylbutyl)amino)-3-hydroxybutanoate (**5 cc**)*

The title compound was prepared starting from compounds **3c** and **2** (R_1 = neopentyl) following the general procedure of **5**. Yield MCR 60%, hydrolysis 60%, light-yellow oil, mixture of atropoisomers. ^{1}H NMR CD_3OD δ 6.76 (bs, 2Ha), 6.75 (bs, 2Hb), 4.66–4.56 (m, 4Ha,b), 4.38–4.32 (m, 1Ha,b), 4.05–3.94 (m, 1Ha,b), 3.81 (s, 6Ha), 3.80 (s, 6Hb), 3.77 (t, *J* = 6 Hz, 2Ha), 3.71 (t, *J* = 6 Hz, 2Hb), 2.85 (t, *J* = 6 Hz, 2Hb), 2.77 (t, *J* = 6 Hz, 2Ha), 2.66–2.28 (m, 8Ha,b), 1.99–1.91 (m, 1Ha,b), 1.88–1.82 (m, 1Ha,b), 0.89 (bs, 9Hb), 0.88 (bs, 9Ha). ^{13}C NMR CD_3OD δ 172.73, 172.68, 158.68, 149.48, 149.37, 149.32, 149.26, 128.25, 127.79, 126.48, 125.95, 113.11, 112.98, 111.06, 110.86, 68.69, 68.46, 56.56, 56.55, 56.50, 56.47, 53.57, 53.33, 51.43, 51,11, 48.43, 48.28, 48.15, 47.91, 45.09, 44.56, 41.51, 41.35, 31.34, 30.97, 30.56, 30.11, 30.09, 29.71, 28.87, 25.98. Anal. Calcd for $C_{26}H_{39}N_6NaO_6$: C, 56.31; H, 7.09; N, 15.15. Found: C, 56.18; H, 7.08; N, 15.13.

*4.7.27. Sodium 4-((cyclopropyl(1-(4-(6,7-dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)-4-oxobutyl)-1H-tetrazol-5-yl)methyl) amino)-3-hydroxybutanoate (**5cd**)*

The title compound was prepared starting from compounds **3c** and **2** (R_1 = cyclopropyl) following the general procedure of **5**. Yield MCR 20%, hydrolysis 50%, light-yellow oil, mixture of atropoisomers. ^{1}H NMR CD_3OD δ 6.85–6.70 (m, 2Ha,b), 4.75–4.62 (m, 2Ha,b), 4.60 (s, 2Ha,b), 4.01–3.92 (m, 1Ha,b), 3.80 (s, 6Ha,b), 3.76 (t, *J* = 6 Hz, 2Ha), 3.70 (t, *J* = 6 Hz, 2Hb), 3.60–3.50 (m, 1Ha,b), 2.84 (t, *J* = 6 Hz, 2Hb), 2.76 (t, *J* = 6 Hz, 2Ha), 2.69–2.39 (m, 4Ha,b), 2.35–2.19 (m, 4Ha,b), 1.40–1.26 (m, 1Ha,b), 0.80–0.66 (m, 1Ha,b), 0.56–0.42 (m, 2Ha,b), 0.32–0.22 (m, 1Ha,b). ^{13}C NMR CD_3OD δ 179.79, 172.76, 172.73, 157.82, 149.50, 149.38, 149.34, 149.28, 128.30, 127.90, 126.57, 126.08, 113.23, 113.12, 111.17, 111.01, 69.71, 69.29, 59.60, 59.18, 59.14, 56.64, 56.61, 56.56, 54.18, 53.81, 48.28, 48.25, 47.93, 45.07, 44.56, 43.30, 43.12, 41.34, 31.03, 30.68, 29.71, 28.86, 26.27, 26.23, 15.88, 5.73, 5.64, 2.98, 2.85. Anal. Calcd for $C_{24}H_{33}N_6NaO_6$: C, 54.95; H, 6.34; N, 16.02. Found: C, 54.80; H, 6.32; N, 15.98.

*4.7.28. Sodium 4-(((1-(4-(6,7-dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)-4-oxobutyl)-1H-tetrazol-5-yl)(thiophen-2-yl)methyl) amino)-3-hydroxybutanoate (**5ce**)*

The title compound was prepared starting from compounds **3c** and **2** (R_1 = 2-thiophenyl) following the general procedure of **5**. Yield MCR 27%, hydrolysis 97%, light-yellow oil, mixture of atropoisomers. ^{1}H NMR CD_3OD δ 7.42–7.35 (m, 1Ha,b), 7.15–7.07 (m, 1Ha,b), 7.00–6.94 (m, 1Ha,b), 6.75 (bs, 2Ha,b), 5.77 (bs, 1Ha,b), 4.61–4.49 (m, 4Ha,b), 4.14–4.05 (m, 1Ha,b), 3.80 (s, 6Ha,b), 3.74 (t, *J* = 6 Hz, 2Ha), 3.60 (t, *J* = 6 Hz, 2Hb), 2.80 (t, *J* = 6 Hz, 2Hb), 2.74 (t, *J* = 6 Hz, 2Ha), 2.72–2.56 (m, 2Ha,b), 2.51–2.32 (m, 4Ha,b), 2.18–2.06 (m, 1Ha,b). ^{13}C NMR CD_3OD δ 172.59, 172.51, 157.38, 149.41, 149.30, 149.24, 149.19, 142.77, 142.57, 128.22, 128.12, 127.97, 127.77, 127.68, 127.61, 126.43, 125.92, 113.02, 112.90, 110.95, 110.79, 69.14, 68.95, 64.99, 56.55, 56.51, 56.47, 56.44, 53.84, 53.69, 53.55, 48.16, 47.83, 45.04, 44.47, 42.20, 41.28, 30.78, 30.36, 29.67, 28.85, 25.86, 25.81. Anal. Calcd for $C_{25}H_{31}N_6NaO_6S$: C, 52.99; H, 5.51; N, 14.83. Found: C, 52.83; H, 5.50; N, 14.80.

*4.7.29. Sodium 4-(benzyl(1-(1-(4-(3,4-dihydroisoquinolin-2(1H)-yl)-4-oxobutyl)-1H-tetrazol-5-yl)-3-methylbutyl)amino)-3-hydroxybutanoate (**16aa**)*

The title compound was prepared starting from compounds **3a**, **15** (R_1 = H) and **2** (R_2 = isobutyl**)** following the general procedure of **16**. Yield MCR 22%, hydrolysis 69%, colorless oil, mixture of atropoisomers. ^{1}H NMR CD_3OD δ 7.36–7.26 (m, 4Ha,b), 7.23–7.13 (m, 5Ha,b), 4.65 (s, 2Ha), 4.62 (s, 2Hb), 4.36–4.21 (m, 3Ha,b), 4.21–4.08 (m, 1Ha), 4.08–3.99 (m, 1Hb), 3.81–3.65 (m, 4Ha,b), 2.92 (t, *J* = 6 Hz, 2Ha) 2.84 (t, *J* = 6 Hz, 2Hb), 2.73–2.44 (m, 2Ha,b), 2.43–2.37 (m, 2Ha,b), 2.22–1.83 (m, 6Ha,b), 1.56–1.40 (m, 1Ha,b), 0.94 (d, *J* = 6.4 Hz, 6Ha), 0.89 (d, *J* = 6.8 Hz, 3Hb), 0.88 (d, 6.4 Hz, 3Hb). ^{13}C NMR CD_3OD δ 172.53, 156.55, 156.41, 140.29, 136.17, 135.75, 134.45, 133.99, 130.74, 130.70, 129.67, 129.56, 129.52, 129.39,128.58, 128.54, 128.01, 127.76, 127.58, 127.50, 127.47, 127.28, 69.36, 68.37, 56.88, 56.72, 56.51, 56.35, 53.31, 52.93, 48.19, 47.93, 47.72, 47.63, 45.35, 44.45, 42.28, 42.16, 41.32, 35.15, 35.01, 34.72, 34.61, 31.06, 30.72, 30.21, 29.37, 26.45, 26.01, 25.95, 25.87, 25.83, 23.82, 23.69, 22.36, 22.29. Anal. Calcd for $C_{30}H_{39}N_6NaO_4$: C, 63.14; H, 6.89; N, 14.73. Found: C, 62.96; H, 6.87; N, 14.69.

*4.7.30. Sodium 4-((1-(1-(4-(3,4-dihydroisoquinolin-2(1H)-yl)-4-oxobutyl)-1H-tetrazol-5-yl)-3-methylbutyl)(4-fluorobenzyl) amino)-3-hydroxybutanoate (**16 ab**)*

The title compound was prepared starting from compounds **3a**, **15** (R_1 = F) and **2** (R_2 = isobutyl**)** following the general procedure of **16**. Yield MCR 24%, hydrolysis 56%, colorless oil, mixture of atropoisomers. ^{1}H NMR CD_3OD δ 7.43–6.99 (m, 8Ha-c), 4.66 (bs, 2Ha), 4.65 (bs, 2Hb,c), 4.44–4.29 (m, 3Ha-c), 4.18–4.10 (m, 1Hc), 4.10–3.99 (m, 1Ha), 3.99–3.87 (m, 1Hb), 3.80–3.66 (m, 4Ha-c), 2.92 (t, J = 5.6 HZ, 2Ha), 2.86–2.78 (m, 2Hb,c), 2.71–2.60 (m, 1Ha-c), 2.54–2.44 (m, 3Ha-c), 2.32–1.83 (m, 5Ha-c), 1.56–1.44 (m, 1Ha-c), 0.94 (d, *J* = 6.8 Hz, 3Ha-c), 0.90 (d, *J* = 6.8Hz, 3Ha), 0.89 (d, *J* = 6.4 Hz, 3Hc), 0.88 (d, *J* = 6.8 Hz, 3Hb). ^{13}C NMR CD_3OD δ 172.56, 164.74, 162.31, 156.57, 156.45, 136.46, 136.17, 135.77, 134.43, 133.97, 132.30, 132.22, 129.62, 129.40, 127.96, 127.74, 127.56, 127.48, 127.32, 116.21, 116.00, 69.96, 68.78, 59.05, 57.29, 56.97, 55.27, 53.78, 53.07, 48.13, 48.02, 47.78, 45.35, 44.45, 44.31, 44.12, 43.22, 41.30, 35.34, 34.99, 31.06, 30.73, 30.48, 30.20, 29.39, 26.45, 26.08, 25.91, 25.76, 23.79, 23.66, 22.37, 22.27. Anal. Calcd for $C_{30}H_{38}FN_6NaO_4$: C, 61.21; H, 6.51; N, 14.28. Found: C, 61.00; H, 6.49; N, 14.23.

*4.7.31. Sodium 4-((1-(1-(4-(3,4-dihydroisoquinolin-2(1H)-yl)-4-oxobutyl)-1H-tetrazol-5-yl)-3,3-dimethylbutyl)(benzyl)amino)-3-hydroxybutanoate (**16ac**)*

The title compound was prepared starting from compounds **3a**, **15** (R_1 = H) and **2** (R_2 = neopentyl**)** following the general procedure of **16**. Yield MCR 92%, hydrolysis 40%, colorless oil, mixture of atropoisomers. ^{1}H NMR CD_3OD δ 7.33–7.10 (m, 9Ha,b), 4.64 (bs, 2Ha), 4.60 (bs, 2Hb), 4.29–3.90 (m, 4Ha,b), 3.83–3.56 (m, 4Ha,b), 2.88 (t, *J* = 6 Hz, 2Ha), 2.81 (t, *J* = 6Hz, 2Hb), 2.70–1.74 (m, 10 Ha,b), 0.80 (bs, 9Ha,b). ^{13}C NMR CD_3OD δ 177.05, 172.33, 172.21, 157.27, 157.19, 140.30, 136.09, 135.64, 134.36, 133.88, 130.67, 129.68, 129.53, 129.48, 129.37, 128.59, 128.53, 128.01, 127.75, 127.57, 127.48, 127.26, 68.91, 67.86, 56.74, 56.53, 56.33, 56.19, 51.39, 51.32, 48.10, 47.45, 47.31, 45.31, 44.36, 44.33, 41.54, 41.32, 41.28, 39.18, 39.13, 38.87, 38.82, 30.97, 30.90, 30.84, 30.63, 30.59, 30.29, 30.12, 29.31, 25.69. Anal. Calcd for $C_{31}H_{41}N_6NaO_4$: C, 63.68; H, 7.07; N, 14.37. Found: C, 63.48; H, 7.04; N, 14.31.

*4.7.32. Sodium (3R)-4-((1-(1-(4-(3,4-dihydroisoquinolin-2(1H)-yl)-4-oxobutyl)-1H-tetrazol-5-yl)-3,3-dimethylbutyl)(benzyl) amino)-3-hydroxybutanoate (**(3R)16ac**)*

The title compound was prepared starting from compounds **3a**, the enantiopure compound **15** (*3R*) (R_1 = H), and **2** (R_2 = neopentyl) following the general procedure of **16**. Yield MCR 68%, hydrolysis 67%, colorless oil, mixture of atropoisomers. ^{1}H NMR CD_3OD δ 7.37–7.13 (m, 9Ha,b), 4.67 (bs, 2Ha), 4.64 (bs, 2Hb), 4.30–3.86 (m, 4Ha,b), 3.86–3.53 (m, 4Ha,b), 2.92 (t, J = 6 Hz, 2Ha), 2.85 (t, J = 6Hz, 2Hb), 2.72–1.70 (m, 10 Ha,b), 0.82 (bs, 9Ha,b). ^{13}C NMR CD_3OD δ 176.99, 172.49, 172.39, 157.41, 157.31, 140.42, 136.19, 135.73, 134.47, 133.98, 130.69, 129.70, 129.57, 129.52, 129.39, 128.62, 128.56, 128.06, 127.79, 127.62, 127.50, 127.28, 69.04, 68.01, 56.81, 56.63, 56.50, 56.35, 51.57, 51.51, 48.19, 47.52, 47.40, 45.38, 44.45, 44.44, 41.56, 41.38, 41.27, 39.31, 39.26, 38.99, 38.94, 31.02, 30.98, 30.93, 30.87, 30.67, 30.64, 30.27, 30.19, 29.36, 25.73. Anal. Calcd for $C_{31}H_{41}N_6NaO_4$: C, 63.68; H, 7.07; N, 14.37. Found: C, 63.51; H, 7.05; N, 14.32.

*4.7.33. Sodium 4-((1-(1-(4-(3,4-dihydroisoquinolin-2(1H)-yl)-4-oxobutyl)-1H-tetrazol-5-yl)-3,3-dimethylbutyl)(4-fluorobenzyl) amino)-3-hydroxybutanoate (**16ad**)*

The title compound was prepared starting from compounds **3a**, **15** (R_1 = F) and **2** (R_2 = neopentyl**)** following the general procedure of **16**. Yield MCR 49%, hydrolysis 81%, colorless oil, mixture of atropoisomers. ^{1}H NMR CD_3OD δ 7.31–7.22 (m, 2Ha,b), 7.21–7.10 (m, 4Ha,b), 7.06–6.97 (m, 2Ha,b), 4.66 (bs, 2Ha), 4.63 (bs, 2Hb), 4.37–4.14 (m, 3Ha,b), 4.03–3.86 (m, 1Ha,b), 3.82–3.58 (m, 4Ha,b), 2.90 (t, J = 6 Hz, 2Ha), 2.83 (t, J = 6 Hz, 2Hb), 2.72–2.39 (m, 5Ha,b), 2.26–1.79 (m, 5Ha,b), 0.81 (bs, 9Ha,b). ^{13}C NMR CD_3OD δ 176.18, 172.44, 172.36, 164.73, 162.29, 157.36, 157.27, 136.44, 136.42, 136.15, 135.70, 134.40, 133.87, 132.29, 132.21, 129.61, 129.35, 127.98, 127.74, 127.56, 127.47, 127.26, 116.27, 116.20, 116.05, 116.00, 69.04, 67.94, 56.78, 56.49, 55.88, 55.71, 51.91, 51.84, 48.09, 47.55, 47.45, 45.35, 44.40, 44.38, 41.42, 41.31, 41.07, 39.47, 39.20, 30.90, 30.83, 30.62, 30.58, 30.22, 30.15, 29.35, 25.77. Anal. Calcd for $C_{31}H_{40}FN_6NaO_4$: C, 61.78; H, 6.69; N, 13.94. Found: C, 61.60; H, 6.67; N, 13.89.

4.8. Caspase-1 inhibition assays (provided by reaction biology CRO)

The caspase-1 assay protocol is based on the cleavage of substrate YVAD-AFC (AFC: 7-amino-4-trifluoromethyl coumarin). YVAD-AFC emits blue light (Em = 400 nm); upon cleavage of the substrate by caspase-1, free AFC emits a yellow-green fluorescence (Ex/Em = 400/505 nm). Comparison of the fluorescence from a treated sample with an untreated control allows determination of the fold increase in caspase-1 activity. Compounds exhibit no fluorescent background that could interfere with the assay. All synthesized compounds were tested 10-dose IC50 with 3-fold serial dilution starting at 100 μM or 10 μM against caspase-1. Control compounds were tested in a 10-dose IC50 with 3-fold serial dilution starting at 10 μM.

4.9. Cell line, differentiation, LPS stimulation

The U937 cells, a human histiocytic lymphoma, were obtained from the American Type Culture Collection (ATCC, Manassas, VA, U.S.A.). The cells were cultured in a humidified incubator with 5% CO_2 at 37 °C, in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Vienna, Austria), 100 U ml^{-1} penicillin and 0.1 mg ml^{-1} streptomycin (Invitrogen), hereafter called Complete Medium (CM).

As described in literature [25], to differentiate the U937 monocytes into macrophages, the cells were seeded in CM in 24 well plates treated with 50 μg/ml phorbol-12-myristate-13-acetate (PMA, SIGMA) for 24h. To induce IL-1β production the U937 differentiated cells were treated with 1 μg/ml of LPS (SIGMA) for 4h [26].

4.10. U937 cell growth inhibition

The effect of **16aa** and **5ae** were evaluated on cell viability of U937 cells. The cells were seeded in 98-well plates with 10000 cells per well and treated with different concentration of **16aa** and **5ae** ranging from 100 μM to 1 nM for 48 h. U937 were also treated with RPM11640 containing DMSO at the same concentration used to dilute **16aa** and **5ae** at the beginning and in the following dilutions. After incubation with **16aa, 5ae** or DMSO an MTT test has been performed to evaluate cells viability. Briefly, after 2 days of culture, 0.1 mg of MTT (in 20 μl of PBS) was added to each well and cells were incubated at 37 °C for 4 h. Cells were then lysed, and the absorbance was read at 595 nm using a microplate reader.

4.11. IL-1β evaluation in the supernatants

Frozen culture SN of cells treated with LPS and **16aa** or **5ae** were thawed and immediately tested for the presence of human IL-1β. The test was carried out by ELISA quantitative sandwich enzyme immunoassay technique (ELISA kit Quantikine, Human Il-1β/Il-1F2 immunoassay, R&D Systems, Minneapolis, USA) specific for natural and recombinant human IL-1β.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix B. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmech.2021.114002.

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