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Arginine Analogues Incorporating Carboxylate Bioisosteric Functions are Micromolar Inhibitors of Human Recombinant DDAH-1

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Dimethylarginine dimethylaminohydrolase (DDAH) is a key enzyme involved in the metabolism of asymmetric dimethylarginine (ADMA) and N-monomethyl arginine (NMMA), which are endogenous inhibitors of the nitric oxide synthase (NOS) family of enzymes. Two isoforms of DDAH have been identified in humans, DDAH-1 and DDAH-2. DDAH-1 inhibition represents a promising strategy to limit the overproduction of NO in pathological states without affecting the homeostatic role of this important messenger molecule. Here we describe the design and synthesis of 12 novel DDAH-1 inhibitors and report their derived kinetic parameters, IC50 and K. Arginine analogue 10a, characterized by an acylsulfonamide isosteric replacement of the carboxylate, showed a 13-fold greater inhibitory potential relative to the known DDAH-1 inhibitor, L-257. Compound 10a was utilized to study the putative binding interactions of human DDAH-1 inhibition using molecular dynamics simulations. The latter suggests that several stabilizing interactions occur in the DDAH-1 active-site, providing structural insights for the enhanced inhibitory potential demonstrated by in vitro inhibition studies.

Introduction

Nitric Oxide (NO) and its related pathways represent an important target in medicinal chemistry not only due to its ability to act as a key physiological messenger and modulator, but also due to the growing evidence identifying its role in the onset and progression of several pathological states. Under physiological conditions NO is synthesized in low, tightly regulated concentrations in order to modulate a number of physiological functions, including: vascular tone and blood flow, platelet and leukocytes cell adhesion, and the long-term potentiation and mediation of behavioural activity. Impairment of NO synthesis is commonly associated with pulmonary and systemic hypertension, kidney disease, erectile dysfunction, pre-eclampsia, and cerebrovascular disease. By contrast, excessive NO concentrations are observed in cases of septic shock, migraine, pulmonary fibrosis, inflammatory diseases, tumour angiogenesis, and neurodegenerative disorders. NO is synthesized through a five-electron oxidative reaction catalyzed by one of the three nitric oxide synthase isoforms (nNOS, eNOS, and iNOS), each with varying tissue distribution. Utilizing NADPH and molecular oxygen (O2), NO is harboured upon the conversion of endogenous L-arginine into L-citrulline (Figure 1).

The methylated arginines, namely asymmetric dimethyl arginine (ADMA) and N-monomethyl arginine (NMMA), are competitive endogenous inhibitors of all three NOS isoforms (Figure 1). ADMA and NMMA are released upon the proteolytic cleavage of proteins methylated by Type-1 protein arginine methyl transferases (PRMTs). The metabolic clearance of ADMA and NMMA is primarily mediated by the dimethylarginine dimethylaminohydrolase (DDAH) family of enzymes which hydrolyse methylated arginines to L-citrulline.

Figure 1. Schematic diagram highlighting the overlap between DDAH and NOS biochemical pathways.
and dimethylamine, or monomethylamine, respectively.\textsuperscript{1,26} Two DDAH isoforms have been identified in humans, DDAH-1 and DDAH-2, which share approximately 62% protein sequence homology yet vary in terms of their tissue distribution.\textsuperscript{27} Both are reported to require chelation with Zn\textsuperscript{2+} and are primarily localized to the cytoplasm, however a hydrophobic secondary motif additionally enables association with lipid membranes, such as the mitochondrial membrane.\textsuperscript{28,29} DDAH-1 is primarily associated with the metabolism of ADMA and NMMA, whereas the biochemical role of DDAH-2 remains unclear. Moreover, literature reports suggest that methylated arginines are not DDAH-2 substrates. Since DDAH plays a key role in the regulation of NO and arginases are not DDAH-2 substrates.

Results and discussion

Inhibitor Design and Synthesis
Among the hDDAH-1 inhibitors reported to date, L-257 displayed the highest selectivity for hDDAH-1, with no inhibitory effect on the NOS and arginase families of enzymes.\textsuperscript{34,36} Due to these favourable characteristics, the chemical scaffold of L-257 was adopted for the synthesis of a small library of two different classes of novel potential hDDAH-1 inhibitors.\textsuperscript{36} L-257 side-chain derivatives and L-257 carboxybetaine bioisosteres, 1,2,3-Triazoles structurally unrelated with L-257 were also investigated in this work.

L-257 side-chain derivatives

Initial modifications introduced alterations to the side-chain, as in compounds \textbf{3a-f (Scheme 1)}, which were obtained from the commercially available carboxamidine 2 and the corresponding alcohols \textbf{1a-f under Mitsunobu conditions,}\textsuperscript{37} using DIAD and Ph\textsubscript{3}P in THF. Products \textbf{3a-f} were isolated in 47-99% yields by column chromatography. Guanidination of Boc-Orn-OtBu hydrochloride 4 with \textbf{3a-f} using DIPEA as a base and subsequent Boc-deprotection in TFA/DCM (1:1 v/v), gave the \textit{N}-monoalkylated-L-arginines \textbf{6a-e} in 69-99% yields (Scheme 1).

Figure 2. \textsuperscript{22,32} Current DDAH inhibitors reported in the literature with their IC\textsubscript{50} values.
To investigate the effect of side-chain alteration at the 2-methoxyethyl end of the L-257 scaffolds, five compounds 6a-e were synthesized bearing diverse substituents. In compound 6a a 2-(methylamino)ethyl group was introduced to determine the effect of substituting the oxygen with nitrogen. To determine the effect of a modified terminal amino group, compound 6b carrying a 2-(dimethylamino)ethyl side-chain was designed. Furthermore, to explore the role of hydrophobic interactions and π-stacking in hDDAH-1 inhibition, compound 6c with a 2-(methylthio)ethyl group and compound 6d bearing a 3-benzylguanidino substituent were designed, respectively. Finally, compound 6e incorporated a 2-fluoroethyl side-chain to investigate the impact of altered electronegativity.

L-257 bioisosteres

Analouges 10a-c (Scheme 2) and 14a,b (Scheme 3) originated from bioisosteric replacement of the carboxylate group on the L-257 scaffold. Compound 10a was designed by replacing the carboxylate with a methyl sulfonimide. The overall geometrical topology and acidity of methyl sulfonimides are similar to the carboxylate, however they are metabolically more resistant, with the potential to form strong polar interactions within the hDDAH-1 active-site. Compounds 10b and 10c are the corresponding O-methyl-hydroxamate and hydroxamate analogues of L-257, respectively. These modifications were introduced to investigate the effect of decreasing the acidity of the carboxylate group by substitution with a functional group characterized by a higher pKₐ (8-9). Z-Orn(Boc)-OH 7 (Scheme 2) was used as starting material for the synthesis of 10a-c. Deprotection of the side-chain Boc-amino group using a solution of HCl (4N) in 1,4-dioxane and guanidination with 3f, followed by full deprotection of the resulting derivatives 9a-c afforded the desired products 10a-c in 22-56% yields.

Analogues 14a and 14b (Scheme 3) incorporated azole bioisosteric groups and were produced from the corresponding primary amide 11 and the nitrile 12 – obtained by TFAA-promoted hydration – using Z-Orn(Boc)-OH 7 as starting material. The guanidine group was introduced after cleavage of the side-chain N-Boc group of 12, followed by reaction with the same Mitsunobu product 3f (Scheme 1) used to synthesize L-257. The resulting nitrile derivative 13 was reacted with sodium azide according to the procedure described by Demko and Sharpless. The resulting tetrazole was fully deprotected using TFA and TFMSA, affording compound 14a. The nitrile 13 was also converted into the 5-oxo-1,2,4-oxadiazole derivative using hydroxylamine hydrochloride. Final guanidine deprotection with TFA provided the free oxadiazolone product 14b. The rationale for introducing a tetrazole ring to generate compound 14a was to replace the carboxylic acid function with a bioisostere having similar acidity (pKₐ 4.5-4.9). This substitution is known to exhibit a 10-fold increase in lipophilicity, greater metabolic resistance, and higher number of hydrogen bond acceptors. Alternatively, the 5-oxo-1,2,4-oxadiazole functional group (compound 14b) exhibits

Scheme 2. L-257 linear bioisosteres synthesis. Reaction conditions: a. CDI, DBU, methanesulfonamide, THF, rt, 7 h, 51%; b. HATU, DIPEA, O-methylhydroxylamine hydrochloride, THF, 0 °C rt, 16 h, 86%; c. HATU, DIPEA, O-benzylhydroxylamine, THF, 0 °C rt, 16 h, 57%; d. HCl/dioxane (4N), DCM, rt, 30 min, 99%; e. DIPEA, DCM, rt, 24 h, 22-99%; f. TFA, TFMSA, rt, 1 h, 99%.

Scheme 3. Azole bioisosteres synthesis. Reaction conditions: α. BuOCOCI, N-methylmorpholine, NH₄OH (30%), THF, -10 °C rt, 3 h, 99%; b. TFAA, TEA, THF, 0 °C rt, 16 h, 47%; c. HCl/dioxane (4N), DCM, rt, 30 min, 99%; d. DIPEA, DCM, rt, 24 h, 22-99%; e. NaH, ZnBr₂, water/2-propanol, reflux, 16 h, 30%; f. TFA, DCM, rt, 3 h, 99% g. 1) NH₄OH/HCl, NaHCO₃, DMSO, reflux, 16 h; 2) CDI, DBU, THF, reflux, 30 min, 73%.
reduced acidity (pKa 6-7)\textsuperscript{47} and increased lipophilicity,\textsuperscript{48} relative to the carboxylate group.

1,2,3-triazoles

The 1,2,3-triazoles 19 and 24 (Scheme 4), were synthesized utilizing Cu\textsuperscript{-}catalyzed click chemistry.\textsuperscript{49} The rationale for generating these triazoles is based on the work of Linsky and Fast,\textsuperscript{50} who identified 4-halopyridines and benzimidazoles as key functions for the development of novel DDAH inhibitors, although 19 and 24 are structurally unrelated to ADMA and NMMA. Interestingly, in the PaDDAH active-site, the 4-halopyridines exhibited covalent binding at the enzyme’s catalytic cysteine, whereas the benzimidazoles were shown to be weak reversible inhibitors, occupying a region of the active-site usually accommodating the substrate’s side-chain. We therefore hypothesized that connecting these two functions through a triazole linker may improve the inhibitory potential of the resulting compounds. Compound 19 was synthesized by cycloaddition between the alkyne 18, obtained by Sonogashira C-C coupling from 2-Bromo-4-chloropyridine 17,\textsuperscript{51} and azide 16, obtained from 1-H-benzimidazole-2-methanol 15 by reaction with diphenylphosphoryl azide\textsuperscript{52} (Scheme 4). Similarly, triazole 24 was synthesized by reacting the alkyne 21, obtained from 2-bromo-1-H-benzimidazole 20 under Sonogashira conditions,\textsuperscript{53} and azide 23 resulting from the reaction between 4-chloro-2-(hydroxymethyl)pyridine 22 and diphenylphosphoryl azide\textsuperscript{53} (Scheme 4).

hDDAH-1 inhibition

All the novel compounds described herein were assessed along with L-257 for their inhibitory potential against hDDAH-1. Recombinant hDDAH-1 was expressed in HEK293T cells according to the procedure described by Lewis et al.\textsuperscript{54} In vitro pharmacokinetic parameters of hDDAH-1 inhibition were derived by detecting L-citrulline formation via UPLC-MS\textsuperscript{55} (see Experimental section).

Inhibition experiments separately comprised each new compound and L-257 (0-1 mM) with ADMA (100 μM) as the substrate. Reactions were initiated by the addition of pre-warmed substrate (37 °C) and allowed to incubate with shaking at 37 °C for 60 min. Deuterated d\textsubscript{2}-L-citrulline (5 mg/L) was used as internal standard. Reactions were terminated by the addition of 200 μL of ice-cold acetic acid 4% in methanol and prepared for analysis by diluting (3:10 v/v) in mobile phase. Incubation reactions utilized five concentrations of inhibitor (0, 0.1, 1, 10, 100 and 1000 μM or 500 μM) in screening studies and five concentrations in the range of 0-75 μM in kinetic analyses.

Inhibition using the L-257 scaffold

In our hands, L-257 resulted in 83% inhibition at a concentration of 1 mM and the IC\textsubscript{50} value was calculated to be 31 μM, with a K\textsubscript{i} of 13 μM.

Inhibition using L-257 side-chain derivatives

Modification of the L-257 methoxyethyl side-chain reduced the inhibitory potential (Table 1). Interestingly, three derivatives (6c-e) gave significant inhibition at concentrations of 1 mM, yet none resulted in low IC\textsubscript{50} values. The best inhibitors of this category were compounds 6c and 6d in which the methoxy group of L-257 was substituted with a methylthio and a fluorine group, respectively. Compound 6c induced 80% of inhibition at 1 mM, relative to control experiments, and resulted in an IC\textsubscript{50} of 408 μM. The IC\textsubscript{50} value for 6d was calculated to be 379 μM, with a percent inhibition of 67% at 1 mM (Table 1).

Scheme 4. Triazoles synthesis. a. DPPA, DBU, toluene, rt, 16 h, 55-85%; b. (Ph\textsubscript{3}P)\textsubscript{2}PdCl\textsubscript{2}, CuI, TEA, ethynylTMS, 80 °C, 24 h, 48%; c. CuI, sodium ascorbate, TEA, DCM, rt, 24 h, 30-65%; d. (Ph\textsubscript{3}P)\textsubscript{2}PdCl\textsubscript{2}, CuI, TIPS-acetylene, DMF, 80 °C, 72 h, 30%.

Table 1. Inhibitory potentials of the L-257 side-chain derivatives

<table>
<thead>
<tr>
<th>Compound</th>
<th>R-</th>
<th>Inhibition at 1 mM (%)</th>
<th>IC\textsubscript{50} (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6a</td>
<td></td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>6b</td>
<td></td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>6c</td>
<td></td>
<td>80</td>
<td>408±19</td>
</tr>
<tr>
<td>6d</td>
<td></td>
<td>67</td>
<td>379±5</td>
</tr>
<tr>
<td>6e</td>
<td></td>
<td>55</td>
<td>866±3</td>
</tr>
</tbody>
</table>

Inhibition using 1,2,3-triazoles

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Low inhibitory potential was observed with the two 1,2,3-triazoles 19 and 24 (Table 2). Both compounds were tested at the maximal concentration of 0.5 mM (instead of 1 mM), due to the poor solubility of these compounds. Compound 19 resulted in 9% inhibition at 0.5 mM and the IC₅₀ value for this molecule was therefore not determined. Compound 24 exhibited greater inhibitory potential with an IC₅₀ of 422 μM and 57% inhibition at 0.5 mM.

Table 2. Inhibitory potentials of the 1,2,3-triazoles

<table>
<thead>
<tr>
<th>Compound</th>
<th>Inhibition at 0.5 mM (%)</th>
<th>IC₅₀ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td>9</td>
<td>-</td>
</tr>
<tr>
<td>24</td>
<td>57</td>
<td>422±4</td>
</tr>
</tbody>
</table>

* Poor solubility at 1 mM

### Inhibition using L-257 bioisosteres

Interestingly, a different trend was observed with L-257 carboxylate bioisosteres (Table 3). Significantly enhanced inhibitory potential was exhibited by the acylsulfonamide 10a that reduced L-citrulline formation of 98% at a concentration of 1 mM, relative to control experiments. The IC₅₀ value determined for this compound was 3 μM. Additionally, the oxadiazolone 14b was also found to be effective, with 95% inhibition at a concentration of 1 mM. The IC₅₀ value derived for this bioisostere was 18 μM.

Compound 14a exhibited 91% inhibition at a concentration of 1 mM, relative to control experiments, and its derived IC₅₀ value was 34 μM. Thus, although 14a was less potent than 10a and 14b, it was still essentially equipotent to the reference compound, L-257.

Noteworthy, the inhibitory potential of these L-257 analogues was significantly decreased when the bioisosteric replacement was less acidic than the carboxylate, such as the hydroxamic group (compound 10b) or the O-methyl-hydroxamic group (compound 10c). This suggests that electrostatic interactions with positively charged residues of the hDDAH-1 active-site may facilitate the inhibitor binding.

Table 3. Inhibitory potentials of the L-257 bioisosteres

<table>
<thead>
<tr>
<th>Compound</th>
<th>R-</th>
<th>Inhibition at 1 mM (%)</th>
<th>IC₅₀ (μM)</th>
<th>Kᵢ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10a</td>
<td></td>
<td>98</td>
<td>3±3</td>
<td>1±0</td>
</tr>
<tr>
<td>10b</td>
<td></td>
<td>79</td>
<td>230±2</td>
<td>-</td>
</tr>
<tr>
<td>10c</td>
<td></td>
<td>84</td>
<td>131±6</td>
<td>-</td>
</tr>
<tr>
<td>14a</td>
<td></td>
<td>91</td>
<td>34±7</td>
<td>14±1</td>
</tr>
<tr>
<td>14b</td>
<td></td>
<td>95</td>
<td>18±7</td>
<td>7±1</td>
</tr>
</tbody>
</table>
A more detailed study of the kinetics of inhibition using compound 10a (Figure 3) and 14a-b with ADMA as the substrate showed agreement with the competitive model of inhibition with a $K_i$ of 1 μM, 14 μM and 7 μM, respectively.

**Molecular Dynamics Simulations**

To gain atomic-level insights into the mechanism of binding to hDDAH-1 we performed molecular dynamics (MD) simulations\(^5\) on the most potent inhibitor, 10a. A 50ns MD simulation showed that inhibitor 10a remains stable within the hDDAH-1 active-site, blocking the access of substrates to the catalytic cysteine (Cys273).

The X-ray structure of L-257 bound to hDDAH-1\(^5\) identifies the hydrogen bonds involving the ligand's carboxamide nitrogen with the receptor's Asp72 side-chain and Leu29 main-chain oxygen atoms. These interactions are reproduced in the MD simulations of L-257 (data not shown). The same interactions are observed in the MD simulations of compound 10a bound to hDDAH-1 (Figure 4). Furthermore, the 2-methoxyethyl and the guanidine groups of compound 10a show polar and hydrophobic interactions with the side-chains of Ser31, Glu77, Ser175, Asn220, and Leu270 and main chain atoms of Arg30, in analogy with the L-257 complex.

Comparison of the X-ray crystal structure of hDDAH-1 bound to L-citrulline and L-257 shows that the Arg144 side-chain may adopt different conformational states, suggesting inhibitor-induced alterations within the hDDAH-1 active-site. In line with this observation, MD simulations with 10a showed that the methyl sulfonamide group exploits a new binding region within the hDDAH-1 binding-site due to a reorganization of the Arg144 side-chain. The majority of interactions formed by the methyl sulfonamide group within this binding region are mediated via main-chain atoms of residues from helix 4. More specifically, one of the sulfonyl O atoms of compound 10a forms four hydrogen bonds: with the main-chain NH of Pro95 and Ser96, with the side-chain OH of Ser96, and with the side-chain NH of Arg97. Interestingly, the first two interactions are not observed in hDDAH-1 bound to L-257 (neither in the crystal structure nor in MD simulations), and are the likely reason for the higher inhibitory potency of 10a relative to L-257. The second oxygen of the sulfonyl group also acts as a hydrogen-bond acceptor with the side-chain NH of Arg144.

Crystal structures show that the ‘lid’ domain (loop region residues between 29 and 39) remains in an open state in the absence of inhibitor, however it adopts a closed conformation in the presence of an inhibitor.\(^5\) One of the key interactions noted in the crystal structure in association to the lid domain is the hydrogen bonding interaction between the carboxamide NH of L-257 and the main-chain O atom of Leu29. The same hydrogen bonding interaction is observed in MD simulations of L-257 and compound 10a. In addition to this interaction, MD simulations identify hydrophobic interactions between the methyl group of the methyl sulfonamide moiety of 10a and the...
side-chain atoms of Leu29 of the ‘lid’ domain. Such interactions further support closure of the ‘lid’ domain, thus corroborating the higher binding potential seen with compound 10a in vitro.

Overall, the enhanced inhibitory effect of compound 10a relative to L-257 is likely due to the network of hydrogen bonding interactions formed by the methyl sulfonamide group.

Conclusions

A total of 12 novel compounds were synthesized and tested for their ability to inhibit hDDAH-1. The kinetic parameters IC50 and Ki were measured. Three compounds: 10a, 14a and 14b, demonstrated strong hDDAH-1 inhibitory potentials, which were higher or comparable to that of the best known inhibitor L-257. Compound 10a (Ki = 1 μM; IC50 = 3 μM), incorporating an acylsulfonamide functional group, and compound 14b (Ki = 7 μM; IC50 = 18 μM), carrying an oxadiazolone moiety, exhibited a 13-fold and 2-fold greater inhibitory potential, respectively, relative to L-257 (Ki = 13 μM; IC50 = 31 μM). The tetrazole substituted compound 14a (Ki = 14 μM; IC50 = 34 μM) exhibited similar inhibitory activity to that of L-257. Owing to their complex synthetic pathways and inhibitory potentials similar to L-257, compound 14a-b may not be ideal candidates for further development. Conversely, compound 10a appears to be a promising hDDAH-1 inhibitor amenable to further structural modifications. MD simulations on this compound revealed that the methyl acylsulfonamide group is involved in a number of stabilizing interactions within the hDDAH-1 active-site, thus providing a mechanistic basis for the observed in vitro kinetic data.

Experimental section

Chemistry

1H (400.13 MHz), 13C (100.58 MHz) and 19F (376.45 MHz) NMR spectra were recorded on a Bruker ADVANCE III spectrometer. For 1H NMR spectra the solvent resonance was employed as the internal standard (CDCl3 δ: 7.26, CD2OD δ: 3.31, D2O δ: 4.79, (CD3)2SO δ: 2.50). 13C NMR spectra were recorded with complete proton decoupling. The following abbreviations are used to describe spin multiplicity: s = singlet, d = doublet, t = triplet, m = multiplet, dd = doublet–doublet, dt = doublet–triplet, ddd = doublet–doublet–doublet, tt = triplet–triplet and br = broad signal. All chemical shifts (δ) are expressed in parts per million (ppm) and coupling constant (J) are given in Hertz (Hz). HPLC–MS experiments were performed on an Agilent Technologies 1200 Series HPLC system equipped with a DAD and a 6120 MS detector composed by an ESI ionization source and a Single Quadrupole mass selective detector using a Analytical C18 RP column (Phenomenex Luna C18(2), 250 mm×4.60 mm, 5 μm, 100 Å). HPLC purifications were performed on the Agilent 1260 system using a semi-preparative C18 RP column (Phenomenex Luna, C18(2), 250 mm×10.00 mm, 5 μm, 100 Å). Specific optical rotation measurements were performed on a PolAAR model 21 at 589 nm and the value were calculated based on the equation [α]D20 = (100α)/(l c) where the concentration c is in g/100 mL and the path length l is 1 dm. All reactions were carried out in oven- or flame-dried glassware under nitrogen atmosphere, unless stated otherwise. All starting materials and reagents were commercially available and they were used as received. Tetrahydrofuran (THF) and dichloromethane (DCM) over molecular sieves and dry N,N'-dimethylformamide (DMF) and dimethylsulfoxide (DMSO) were purchased from Acros Organics and used without further purification. Reactions were magnetically stirred and monitored by TLC on silica gel (60 F254 pre-coated glass plates, 0.25 mm thickness). Visualization was accomplished by irradiation with a UV lamp and/or staining with a ceric ammonium molybdate or KMnO4 solution. Flash chromatography were performed on silica gel (60 Å, particle size 0.040–0.062 mm).

L-257 side-chain derivatives

General method for Mitsunobu reaction (Procedure A)

Diisopropyl azodicarboxylate (DIAD, 1.5 eq) was added dropwise to a stirring solution of N,N'-bis-tert-butoxycarbonylpyrazole-1H-carboxamidine (1 eq), the proper alcohol (1-2 eq) and triphenylphosphine (1.5 eq) in THF (7 mL) at 0 °C. The reaction was stirred overnight on room temperature, concentrated in vacuum and purified by flash column chromatography to achieve the N-alkylated product 3a-f as a colourless oils.

General method for guanidination (Procedure B)

Boc-Orn-OtBu hydrochloride (1 eq) was dissolved in DCM (2 mL) and DIPEA (1.5 eq) was added to the stirring suspension obtaining a velar solution. The desired guanilating reagent obtained with procedure A (1 eq) was then added to the solution and the mixture was stirred for 24 hours at room temperature. After the addition of DCM (10 mL), the organic phase was washed with an aqueous solution of KHSO4 (1M, 2 x 10 mL), water (10 mL), brine (10 mL) and dried over anhydrous sodium sulphate. The solvent was completely evaporated and the residue was purified by flash column chromatography to achieve the guanidino tert-butyl ester 5a-e as white foams.

General method for Boc- and tert-butyl deprotection (Procedure C)

The final deprotection was achieved by stirring at room temperature for 3 hours a solution of the tert-butyl esters 5a-e (1 eq) in TFA/DCM (1:1, 4 mL). The TFA was completely evaporated under a reduced air flow, the deprotected product was washed with n-hexane and precipitated with diethyl ether. A further purification via RP-HPLC was required, achieving the desired products 6a-e as foams in quantitative yield.
tert-Butyl N-((tert-butoxy)carbonyl)[2-(methylamino)ethyl]amino][1H-pyrazol-1-yl)methylidene] carbamate. 3a

(Procedure A) Yield 75% (0.352 g), Rf 0.24 (n-Hexane/AcOEt 80:20).

1H NMR (400 MHz, CDCl3) δ: 7.85 (br, 1H, ArH), 7.61 (br, 1H, ArH), 6.35 (br, 1H, ArH), 3.75 (br, 2H, CH2), 3.50 (br, 2H, CH2), 2.82 (s, 3H, CH3), 1.40 (s, 9H, tBu), 1.35 (s, 9H, tBu), 1.19 (s, 9H, tBu).

13C NMR (100 MHz, CDCl3) δ: 157.4 (CO), 155.5 (CO), 152.2 (CO), 144.6 (CN), 142.8 (C15), 130.0 (C14), 108.9 (C9), 82.9 (CtBu), 82.3 (CtBu), 79.6 (CtBu), 47.4 (CH3 N), 46.4 (NCH3), 34.6 (CH2), 28.3 (tBu), 27.9 (tBu), 27.6 (tBu).

ESI MS m/z: [M+Na]+ calcd for C12H24N2O5 490.3, [M+H]+ calcd for C12H24N2O4 468.3, found (relative intensity) 490.3 (100) [M+Na]+, 468.3 (50) [M+H]+.

tert-Butyl N-((tert-butoxy)carbonyl)[limino][1H-pyrazol-1-yl)methyl][N-2(dimethylamino)ethyl] carbamate. 3b

(Procedure A) Yield 47% (0.180 g), Rf 0.15 (n-Hexane/AcOet 80:20).

1H NMR (400 MHz, CDCl3) δ: 7.93 (s, 1H, ArH), 7.60 (d, J = 1.0 Hz, 1H, ArH), 6.31 (dd, J = 2.6, 1.6 Hz, ArH), 3.76 (t, J = 6.0 Hz, 2H, CH2N), 2.53 (t, J = 6.5 Hz, 2H, NCH3), 2.13 (s, 6H, 2x CH3N), 1.41 (s, 9H, tBu), 1.18 (s, 9H, tBu).

13C NMR (100 MHz, CDCl3) δ: 157.6 (CO), 152.4 (CO), 144.7 (CN), 142.8 (C15), 130.2 (C14), 108.4 (C9), 82.5 (CtBu), 82.0 (CtBu), 57.4 (CH3 N), 46.2 (NCH3), 45.2 (2 x CH3 N), 27.9 (tBu), 27.7 (tBu).

ESI MS m/z: [M+H]+ calcd for C14H26N2O5 382.3, found (relative intensity) 382.2 (100) [M+H]+.

tert-Butyl N-((tert-butoxy)carbonyl)[limino][1H-pyrazol-1-yl)methyl][N-2(methylsulfonyl)ethyl] carbamate. 3c

(Procedure A) Yield 42% (0.162 g), Rf 0.28 (n-Hexane/AcOet 90:10).

1H NMR (400 MHz, CDCl3) δ: 7.94 (s, 1H, ArH), 7.62 (d, J = 1.0 Hz, 1H, ArH), 6.36 (dd, J = 2.7, 1.6 Hz, 1H, ArH), 3.95-3.87 (m, 2H, CH2N), 2.81-2.75 (m, 2H, S CH2), 2.04 (s, 3H, CH3S), 1.42 (s, 9H, tBu), 1.20 (s, 9H, tBu).

13C NMR (100 MHz, CDCl3) δ: 157.4 (CO), 152.2 (CO), 144.6 (CN), 143.0 (C15), 130.1 (C14), 108.9 (C9), 82.8 (CtBu), 82.3 (CtBu), 47.5 (CH3 N), 32.2 (SC2H), 27.9 (tBu), 27.6 (tBu), 15.0 (CH3S).


tert-Butyl N-((tert-butoxy)carbonyl)[limino][1H-pyrazol-1-yl)methyl][N-2fluoroethyl] carbamate. 3d

(Procedure A) Yield 97% (0.345 g), Rf 0.44 (n-Hexane/AcOet 80:20).

1H NMR (400 MHz, CDCl3) δ: 7.94 (s, 1H, ArH), 7.68 (dd, J = 1.5, 0.6 Hz, 1H, ArH), 6.42 (dd, J = 2.8, 1.6 Hz, 1H, ArH), 4.67 (dt, JH-F = 46.9 Hz, JF-H = 5.2 Hz, 2H, FCH2), 4.00 (dt, JHF = 23.5, JFH = 4.6 Hz, 2H, CH2 N), 1.49 (s, 9H, tBu), 1.28 (s, 9H, tBu).

13C NMR (100 MHz, CDCl3) δ: 157.3 (CO), 152.3 (CO), 144.4 (CN), 143.3 (C16), 130.0 (C15), 109.0 (C9), 83.2 (CtBu), 82.5 (CtBu), 81.2 (d, JF-H = 169.5 Hz, FCH2), 48.6 (d, JHF = 22.3 Hz, CH2 N), 27.9 (tBu), 27.7(tBu).

19F NMR (376 MHz, CDCl3) δ: -225.68 (tt, J = 46.9, J = 23.5, 1F).


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1.40 (s, 9H, \text{CH}_3), 3.80 (t, J = 6.9 Hz, 2H, \text{CH}_2N), 3.60 (br, 2H, \text{CH}_2), 2.49 (br, 2H, NCH_2), 1.88-1.72 (m, 1H, \text{CH}_2), 1.66-1.54 (m, 3H, CH2), 1.44 (s, 9H, \text{CH}_3), 1.43 (s, 9H, \text{CH}_2), 1.41 (s, 18H, \text{CH}_2), \text{CH}_3)\). 

13C NMR (100 MHz, DMSO, 80 °C) δ: 171.5 (2xCO), 82.6 (Ct, 28.4 (tBu), 27.9 (tBu), 24.9 (CH_3).

ESI MS m/z: [M+H]⁺ calc for C_{18}H_{32}N_5O_6Na 577.4, found (relative intensity) 577.4 (100) [M+H]⁺.

tert-Butyl 5-[3-benzyl-2,3-bis-(tert-butoxycarbonyl)guanidino]-2-[(tert-butoxycarbonyl)amino]pentanoate. 5c

(Procedure B) Yield 99% (0.601 g), R_f 0.16 (Dichloromethane/Methanol 96:4).

1H NMR (400 MHz, CDCl_3) δ: 7.76 (broad peak, 2H, NH_2), 6.46 (J = 6.3 Hz, 1H, CH), 3.63 (t, J = 5.9 Hz, 2H, CH_N), 3.36-3.22 (m, 4H, 2xNCH_2), 2.67-2.54 (m, 2H, CH_N), 2.36 (s, 3H, CH_3N), 2.26-2.11 (m, 2H, CH_2), 1.91-1.73 (m, 2H, CH_2), 1.42 (s, 9H, tBu), 1.37 (s, 9H, tBu).

13C NMR (100 MHz, CDCl_3) δ: 171.5 (2xCO), 82.6 (Ct, 28.4 (tBu), 27.9 (tBu), 24.9 (CH_3).

ESI MS m/z: [M+H]⁺ calc for C_{18}H_{32}N_5O_6Na 577.4, found (relative intensity) 577.4 (100) [M+H]⁺.

tert-Butyl 5-[3,2-bis-(tert-butoxycarbonyl)-3-[(methylthio)ethyl]guanidino]-2-[(tert-butoxycarbonyl)amino]pentanoate. 5d

(Procedure B) Yield 99% (0.570 g), R_f 0.35 (n-Hexane/AcOEt 80:20).

1H NMR (400 MHz, MeOD) δ: 5.15 (d, J = 7.7 Hz, 1H, NCH_2), 5.06 (m, 1H, CH), 3.75 (t, J = 6.8 Hz, 2H, CH_2), 3.18 (m, 1H, CH), 2.51 (t, J = 5.9 Hz, 2H, CH_N), 2.48 (s, 3H, CH_3), 1.85-1.71 (m, 2H, CH_2), 1.68-1.50 (m, 3H, CH_2), 1.42 (s, 9H, tBu), 1.37 (s, 9H, tBu).

13C NMR (100 MHz, MeOD) δ: 170.6 (CO), 156.3 (CN_3), 52.2 (CH), 47.4 (NCH_2), 40.5 (NCH_3), 37.6 (CH_2N), 32.4 (CH_2N), 27.3 (CH_2), 24.0 (CH_3).

HRMS calcd. for C_{18}H_{32}N_5O_6Na 577.4, found (relative intensity) 577.4 (100) [M+H]⁺, 643.4 (10) [2M+H]⁺.

2-Amino-5-[(2-methylamino)ethyl]guanidino]pentanoic acid. 6a

(Procedure C) Yield 99% (0.452 g).

1H NMR (400 MHz, MeOD) δ: 7.76 (broad peak, 2H, NH_2), 6.46 (broad peak, 1H, NH), 4.01 (t, J = 5.9 Hz, 2H, CH_N), 3.36-3.22 (m, 4H, 2xNCH_2), 2.67-2.54 (m, 2H, CH_N), 2.36 (s, 3H, CH_3N), 2.26-2.11 (m, 2H, CH_2), 1.91-1.73 (m, 2H, CH_2), 1.42 (s, 9H, tBu), 1.37 (s, 9H, tBu).

13C NMR (100 MHz, MeOD) δ: 170.0 (CO), 156.3 (CN_3), 52.2 (CH), 47.4 (NCH_2), 40.5 (NCH_3), 37.6 (CH_2N), 32.4 (CH_2N), 27.3 (CH_2), 24.0 (CH_3).

HRMS calcd. for C_{18}H_{32}N_5O_6Na 577.4, found (relative intensity) 577.4 (100) [M+H]⁺, 643.3 (10) [2M+H]⁺.

2-Amino-5-[(2-(dimethylamino)ethyl]guanidino]pentanoic acid. 6b

(Procedure C) Yield 99% (0.581 g).

1H NMR (400 MHz, MeOD) δ: 3.98 (t, J = 6.3 Hz, 1H, CH), 3.68 (t, J = 6.2 Hz, 2H, CH_N), 3.39 (t, J = 6.2 Hz, 2H, NCH_2), 3.35-3.24 (m, 2H, NCH_2), 2.96 (s, 3H, 2xCH_2N), 2.08-1.92 (m, 2H, CH_2), 1.89-1.71 (m, 2H, CH_2).

13C NMR (100 MHz, MeOD) δ: 170.6 (CO), 156.3 (CN_3), 55.4 (NCH_2), 52.3 (CH), 42.3 (2xCH_2N), 40.5 (NCH_3), 36.3 (CH_2N), 27.2 (CH_2), 24.0 (CH_3).

ESI MS m/z: [M+H]⁺ calc for C_{18}H_{32}N_5O_6Na 577.4, found (relative intensity) 577.4 (100) [M+H]⁺, 643.4 (10) [M+2H]⁺.
HRMS calcd. for C₈H₁₈FN₄O₂: 221.1408, found: 221.1406.

ESI MS m/z: [M+H]+ calcd for C₈H₁₈FN₄O₂ 221.1, found (relative intensity) 221.1 (100) [M+H]+.

HRMS calcd. for C₁₃H₂₁N₄O₂S: 249.1389, found: 249.1387.

HRMS calcd. for C₁₃H₂₁N₄O₂: 265.1659, found: 265.1657.

**Bioisoteres of the L-257**

**General method for guanidination (Procedure D)**

The opportune intermediates 8a-c or 12 (1 eq) was dissolved in DCM (2 ml) and a solution of HCl in 1,4-dioxane (4N, 2 ml) was added. The mixture was stirred at room temperature for 30 minutes and then the solvent was evaporated to dryness. The hydrochloric salt obtained was used crude and redissolved in DCM (2 ml) and the desired guanilating reagent 3f (1 eq) was added to the stirring suspension. DIPEA (1.5 eq) was then added dropwise and in 3 different portions to the solution and the mixture was stirred for 24 hours at room temperature. After the addition of DCM (10 ml), the crude was washed with an aqueous solution of KHSO₄ (1M, 2 x 10 ml), water (10 ml), brine (10 ml) and dried over anhydrous sodium sulphate. The solvent was completely evaporated and the crude was purified by flash column chromatography to achieve the guanidino compounds 9a-o or 13 as a foamy oils.

**Benzy1 tert-buty1 [5-(methoxysulphonamido)-5-oxopentane-1,4-diyldicarbamate. 8a**

To a solution of Z-Orn(Boc)-OH 7 (1 eq) in dry THF (10 ml), CDI (2 eq) was added under nitrogen flow and the solution stirred for 1 hour at room temperature. DBU (1.5 eq) and methane sulphonamide (1.5 eq) were then added and the resulting mixture was stirred overnight at room temperature. The solvent was evaporated and the crude residue redissolved in DCM (10 ml) and washed with 5% citric acid (2x10 ml), water (10 ml) and brine (10 ml). The organic phase was dried over anhydrous sodium sulphate and the solvent evaporated. The crude residue was purified by flash column chromatography (6% Methanol in Dichloromethane) achieving the desired product 8a as a white foam.

Yield 51% (0.225 g), Rᵢ 0.36 (Dichloromethane/Methanol 90:10).

[a]D = + 3.00 (c 1.0, MeOH).

HRMS calcd. for C₁₃H₁₈FN₄O₂S: 221.1408, found: 221.1406.

**Bioisoteres of the L-257**

**General method for deprotection (Procedure E)**

The final deprotection was achieved by stirring at room temperature for 1 hour a solution of compound 9a-c (1 eq), TFMSA (6 eq) and TFA (2 ml) The TFA and TFMSA were completely evaporated under a compressed air flow, the deprotected product was washed with n-Hexane and precipitated with diethyl ether. A further purification via RP-HPLC was required to obtain the desired products 10a-c or 14a-b as a foamy oils in quantitative yield.

**Benzy1 tert-buty1 [5-(methoxysulphonamido)-5-oxopentane-1,4-diyldicarbamate. 8b**

To a solution of Z-Orn(Boc)-OH 7 (1 eq) in dry THF (10 ml), CDI (2 eq) was added under nitrogen flow and the solution stirred for 1 hour at room temperature. DBU (1.5 eq) and methane sulphonamide (1.5 eq) were then added and the resulting mixture was stirred overnight at room temperature. The solvent was evaporated and the crude residue redissolved in DCM (10 ml) and washed with 5% citric acid (2x10 ml), water (10 ml) and brine (10 ml). The organic phase was dried over anhydrous sodium sulphate and the solvent evaporated. The crude residue was purified by flash column chromatography (6% Methanol in Dichloromethane) achieving the desired product 8a as a white foam.

Yield 51% (0.225 g), Rᵢ 0.36 (Dichloromethane/Methanol 90:10).

[a]D = + 3.00 (c 1.0, MeOH).

HRMS calcd. for C₁₃H₁₈FN₄O₂S: 221.1408, found: 221.1406.

**Bioisoteres of the L-257**

**General method for guanidination (Procedure D)**

The opportune intermediates 8a-c or 12 (1 eq) was dissolved in DCM (2 ml) and a solution of HCl in 1,4-dioxane (4N, 2 ml) was added. The mixture was stirred at room temperature for 30 minutes and then the solvent was evaporated to dryness. The hydrochloric salt obtained was used crude and redissolved in DCM (2 ml) and the desired guanilating reagent 3f (1 eq) was added to the stirring suspension. DIPEA (1.5 eq) was then added dropwise and in 3 different portions to the solution and the mixture was stirred for 24 hours at room temperature. After the addition of DCM (10 ml), the crude was washed with an aqueous solution of KHSO₄ (1M, 2 x 10 ml), water (10 ml), brine (10 ml) and dried over anhydrous sodium sulphate. The solvent was completely evaporated and the crude was purified by flash column chromatography to achieve the guanidino compounds 9a-o or 13 as a foamy oils.

**Benzy1 tert-buty1 [5-(methoxysulphonamido)-5-oxopentane-1,4-diyldicarbamate. 8b**

To a solution of Z-Orn(Boc)-OH 7 (1 eq) in dry THF (1.5 ml) at 0 °C, HATU (1 eq) and DIPEA (2 eq) were added and the solution, which immediately turned bright yellow, was stirred for 10 minutes at 0 °C and then added dropwise to a stirring solution of O-methylhydroxylamine hydrochloride (1 eq) and DIPEA (2 eq) in dry THF (1.5 ml) at room temperature. The resulting mixture was stirred overnight at room temperature, then the
solvent was evaporated and the residue redissolved in DCM (10 mL), washed with 0.1 N HCl (10 mL), water (10 mL) and brine (10 mL). The organic phase was dried over anhydrous sodium sulphate and the solvent evaporated achieving a white residue which was used directly in the next step, without further purification.

Yield 86% (0.470 g), Rf 0.26 (Dichloromethane/Methanol 95:5).

1H NMR (400 MHz, CDCl3) δ 10.33 (br, 1H, NH), 7.41-7.23 (m, 5H, ArH), 6.02 (br, 1H, NH), 5.17-4.93 (m, 3H, NH, CH2), 4.24 (br, 1H, CH), 3.70 (s, 3H, OCH3), 3.28-2.97 (m, 2H, NCH2), 1.83-1.61 (m, 2H, CH2), 1.58-1.47 (m, 2H, CH2), 1.41 (s, 9H, tBu).

13C NMR (100 MHz, CDCl3) δ 169.5 (CO), 156.7 (CO), 156.4 (CO), 136.2 (Cα), 128.5 (2xCα), 128.1 (Cα), 127.9 (2xCα), 79.4 (CβBu), 67.0 (CH3), 64.1 (OCH3), 51.4 (CH), 39.2 (NCH2), 30.0 (CH2), 28.4 (tBu), 26.1 (CH3).

ESI MS m/z: [M+Na]+ calcd for C19H23N5O11 418.2, found (relative intensity) 418.2 (100) [M+Na]+.

Benzyl tert-buty1 (5-[benzoxylamo]-5-oxopentane-1,4-diy1)dicarbamate. 8c

To a solution of 3-OH(Boc)-OH 7 (1 eq) in dry THF (1.5 mL) at 0 °C, HATU (1 eq) and DIPEA (2 eq) were added and the solution, which immediately turned bright yellow, was stirred for 10 minutes at 0 °C and then added dropwise to a stirring solution of O-benzylhydroxylamine hydrochloride (1 eq) and DIPEA (2 eq) in dry THF (1.5 mL) at room temperature. The resulting mixture was stirred overnight at room temperature, then the solvent was evaporated and the residue redissolved in DCM (10 mL) washed with 0.1 N HCl (10 mL), water (10 mL) and brine (10 mL). The organic phase was dried over anhydrous sodium sulphate and the solvent evaporated achieving a white residue which was used directly in the next step, without further purification.

Yield 57% (0.269 g), Rf 0.36 (Dichloromethane/Methanol 96:4).

1H NMR (400 MHz, DMSO, 80 °C) δ: 10.88 (s, 1H, NH), 7.46-7.24 (m, 10H, ArH), 6.97 (s, 1H, NH), 6.35 (s, 1H, NH), 5.06 (s, 2H, CH2), 4.81 (s, 2H, CH2), 3.94 (br, 1H, CH), 2.9 (dd, J = 12.8, 6.7 Hz, 2H, NCH2), 1.68-1.54 (m, 5H, CH2), 1.49-1.35 (m, 11H, CH2, tBu).

13C NMR (100 MHz, DMSO) δ: 169.2 (CO), 156.3 (CO), 156.0 (CO), 137.5 (Cα), 136.3 (Cα), 129.3 (4xCα), 128.8 (Cα), 128.7 (Cα), 128.2 (2xCα), 128.1 (2xCα), 77.9 (CβBu), 77.3 (CH2), 65.9 (CH2), 52.7 (CH), 40.7 (NCH2), 29.7 (CH2), 28.7 (tBu), 26.5 (CH3).

ESI MS m/z: [M+Na]+ calcd for C23H29N5O10S 644.3, found (relative intensity) 644.3 (100) [M+Na]+.

Benzyl (10-[(tert-butoxy carbonyl)amino]-11-[(2-methoxyethyl)-14,14-dimethyl-4,12-dioxo-2,13-dioxa-3,9,11-triazapentadec-9-en-5-yl]carbamate. 9b

(Procedure D) Yield 22% (0.132 g), Rf 0.3 (Dichloromethane/Methanol 95:5).

[a]D20 = +1.33 (c 1.5, MeOH).

1H NMR (400 MHz, DMSO) δ: 11.24 (s, 1H, NH), 7.54-7.50 (m, 1H, NH), 7.41-7.26 (m, 5H, ArH), 5.03 (s, 2H, CH2), 3.87-3.78 (m, 1H, CH), 3.43 (br, 4H, OCH2, CH2N), 3.24 (s, 2H, CH2O), 3.10 (br, 2H, NCH2), 2.70 (s, 3H, OCH3), 1.70-1.45 (m, 4H, 2x CH2), 1.39 (s, 9H, tBu), 1.38 (s, 9H, tBu).

13C NMR (100 MHz, DMSO) δ: 168.8 (CO), 156.3 (CO), 152.0 (CO), 137.4 (Cα), 128.8 (2xCα), 128.3 (Cα), 128.2 (2xCα), 79.7 (CβBu), 78.1 (CβBu), 70.3 (OCH3), 65.9 (CH2), 58.5 (CH2O), 52.6 (CH), 47.1 (CH2N), 41.7 (NCH2), 38.7 (OCH3), 29.6 (CH2), 28.4 (tBu), 28.3 (tBu), 25.1 (CH3).

ESI MS m/z: [M+H]+ calcd for C29H46N5O15S 696.3, found (relative intensity) 696.3 (100) [M+H]+.

Benzyl (10-[(tert-butoxy carbonyl)amino]-11-[(2-methoxyethyl)-14,14-dimethyl-4,12-dioxo-1-phenyl-2,13-dioxa-3,9,11-triazapentadec-9-en-5-yl]carbamate. 9c

(Procedure D) Yield 56% (0.376 g), Rf 0.36 (Dichloromethane/Methanol 95:5).

[a]D20 = -2.00 (c 1.0, MeOH).

1H NMR (400 MHz, DMSO) δ: 11.26 (s, 1H, NH), 7.57-7.48 (m, 1H, NH), 7.43-7.26 (m, 10H, ArH), 5.04 (s, 2H, CH2), 4.79 (s, 2H, CH2), 3.93-3.80 (m, 1H, CH), 3.43 (br, 4H, OCH2, CH2N), 3.32 (s, 3H, CH2O), 3.09 (br, 2H, NCH2), 1.67-1.42 (m, 4H, 2xCH2), 1.39 (s, 9H, tBu), 1.37 (s, 9H, tBu).

13C NMR (100 MHz, DMSO) δ: 169.1 (CO), 156.3 (2xCO), 152.5 (CO), 137.5 (Cα), 136.3 (Cα), 129.3 (4xCα), 128.8 (Cα), 128.7 (Cα), 128.3 (2xCα), 128.2 (2xCα), 81.1 (CβBu), 78.1 (CβBu), 77.3 (CH2), 70.3 (NH), 65.9 (CH2), 58.5 (CH2O), 52.7 (CH), 47.1 (OCH3), 41.8 (NCH2), 29.7 (CH2), 28.4 (tBu), 28.3 (tBu), 25.0 (CH3).

ESI MS m/z: [M+H]+ calcd for C34H45N5O17 767.3, found (relative intensity) 767.3 (100) [M+H]+.

2-Amino-5-[3-(2-methoxyethyl)guanidino]-N-(methylsulfonyl)pentanamide. 10a

(Procedure E) Yield 99% (0.437 g).

[a]D20 = +3.54 (c 2.5, MeOH).

1H NMR (400 MHz, D2O) δ: 3.94 (t, J = 6.0 Hz, 1H, CH), 3.53 (t, J = 5.0 Hz, 2H, CH2N), 3.35-3.29 (m, 5H, OCH2, CH2N), 3.25-3.15 (m, 5H, 2xCH2, CH2O), 1.98-1.84 (m, 2H, CH2), 1.74-1.52 (m, 2H, CH2).
11C NMR (100 MHz, D2O) δ: 172.0 (CO), 156.2 (CN2), 70.3 (CH2N), 58.2 (CH2), 54.0 (OCH2), 40.3 (NCH2), 40.2 (CH2O), 27.5 (CH2), 23.4 (CH2).
ESI MS m/z: [M+H]+ calcd for C41H32N7O7S 310.0, found (relative intensity) 310.1 (100) [M+H]+.
HRMS calc'd for C41H32N7O7S: 310.1544, found: 310.1542.

2-Amino-N-methoxy-5-(3-[2-methoxyethyl]guanidino)pentanamide. 10b
(Procedure E) Yield 99% (0.088 g).
[a]D = +10.00 (c 0.5, MeOH).
1H NMR (400 MHz, D2O) δ: 3.79 (t, J = 6.9 Hz, 1H, CH), 3.70 (s, 3H, OCH3), 3.53 (t, J = 5.3 Hz, 2H, CH2N), 3.32 (t, J = 5.3 Hz, 2H, OCH2), 3.19 (t, J = 6.8 Hz, 2H, NCH2), 1.92-1.77 (m, 2H, CH2), 1.64-1.51 (m, 2H, CH2).
13C NMR (100 MHz, D2O) δ: 165.9 (CO), 156.2 (CN2), 70.3 (CH2N), 64.4 (OCH2), 58.2 (CH2O), 50.7 (CH), 41.1 (OCH2), 40.3 (NCH2), 27.7 (CH2), 23.7 (CH3).
ESI MS m/z: [M+H]+ calcd for C11H16N5O2 262.2, found (relative intensity) 262.2 (100) [M+H]+.
HRMS calc'd for C11H16N5O2: 262.1874, found: 262.1872.

2-Amino-N-hydroxy-5-(3-[2-methoxyethyl]guanidino)pentanamide. 10c
(Procedure E) Yield 99% (0.118 g).
[a]D = +14.10 (c 0.8, MeOH).
1H NMR (400 MHz, D2O) δ: 3.79 (t, J = 7.0 Hz, 1H, CH), 3.53 (t, J = 5.0 Hz, 2H, CH2N), 3.36-3.28 (m, 5H, OCH2, CH2O), 3.18 (t, J = 6.8 Hz, 2H, NCH2), 1.94-1.77 (m, 2H, CH2), 1.69-1.46 (m, 2H, CH2).
13C NMR (100 MHz, D2O) δ: 166.2 (CO), 156.2 (CN2), 70.3 (CH2N), 58.2 (CH2O), 50.9 (CH), 41.1 (OCH2), 40.4 (NCH2), 27.8 (CH2), 23.8 (CH3).
ESI MS m/z: [M+H]+ calcd for C11H16N5O2 248.2, found (relative intensity) 248.2 (100) [M+H]+.

Benzyl tert-buty1-(5-amino-5-oxopentan-1-4-diy1)dicarbatate. 12
Z-Orn(Boc)-OH 7 (1 eq) and N-methylmorpholine (1.1 eq) were dissolved in THF (7 ml), the solution was cooled down to -10 °C. Isobutyl chloroformate (1.1 eq) was added dropwise and the mixture was stirred for 20 minutes. An aqueous ammonia solution 30% (6 eq) was then added and the reaction was allowed to warm to room temperature. After 1 h, AcOEt (10 ml) was added. The mixture was washed with an aqueous HCl solution (1N, 10 ml), water (10 ml) and dried over anhydrous sodium sulphate. The solvent was evaporated and the resulting oil was purified by flash column (30% AcOEt in n-Hexane) achieving the desired product 12 as a colourless foamy oil.
Yield 47% (0.162 g), Rf 0.35 (n-Hexane/AcOEt 60:40).
[a]D = -27.00 (c 1.0, MeOH).
1H NMR (400 MHz, CDCl3) δ: 7.42-7.28 (m, 5H, ArH), 5.91 (br, 1H, NH), 4.84 (4H, ArH, 6.7-5.50 (m, 1H, CH), 3.12 (2H, NCH2), 1.90-1.71 (m, 2H, CH2), 1.71-1.52 (m, 2H, CH2), 1.43 (s, 9H, tBu).
13C NMR (100 MHz, CDCl3) δ: 156.2 (CO), 155.5 (CO), 135.8 (CN2), 128.6 (2xC2H5), 128.4 (2xC2H5), 118.7 (CN), 79.5 (CF3), 67.5 (CH2), 42.6 (CH), 39.5 (NCH2), 30.2 (CH2), 28.4 (tBu), 26.2 (CH2).
ESI MS m/z: [M+Na]+ calcd for C27H32N5O4Na 370.2, found (relative intensity) 370.1 (100) [M+Na]+.

(Procedure D) Yield 40% (0.219 g), Rf 0.31 (n-Hexane/AcOEt 50:50).
[a]D = -17.00 (c 1.0, MeOH).
1H NMR (400 MHz, DMSO) δ: 8.21 (d, J = 7.8 Hz, 1H, NH), 7.45-7.31 (m, 5H, ArH), 5.09 (2H, CH2), 4.61 (dd, J = 7.5 Hz, J = 7.5 Hz, 1H, CH), 3.44 (4H, ArH, 2xC2H5), 3.24 (3H, CH3), 3.14 (2H, NCH2), 1.89-1.70 (m, 2H, CH2), 1.69-1.52 (m, 2H, CH2), 1.40 (s, 9H, tBu), 1.38 (9H, tBu).
13C NMR (100 MHz, DMSO) δ: 159.5 (2xCOC), 152.5 (2xCN2), 136.9 (CN2), 128.9 (2xC2H5), 128.5 (2xC2H5), 128.4 (2xC2H5), 120.1 (CN), 81.1 (tBu), 78.1 (tBu), 70.3 (CH3), 66.6 (CH3), 58.5 (CH2O), 47.1 (OCH2), 42.4 (CH), 40.6 (NCH2), 29.6 (CH2), 28.4 (tBu), 28.3 (tBu), 24.7 (CH2).
ESI MS m/z: [M+H]+ calcd for C27H42N7O7 548.3, [M+Na]+ calcd for C27H42N7O7Na 570.3, found (relative intensity) 548.3 (100) [M+H]+, 570.3 (10) [M+Na]+.

1-[4-Amino-4-(1H-tetrazol-5-yl)butyl]-3-[2-methylthoxy]guanidine. 14a
The guanidine nitrite 13 (1 eq) was dissolved in water (10 ml) and 2-propanol (5 ml) then sodium azide (2 eq) and zinc bromide (0.5 eq) were added. The reaction was refluxed overnight, then the solvent evaporated and the residue was...
dissolved in TFA (2 mL) and TFMSA (12 eq). The reaction was stirred for 1 hour at room temperature, the acids were evaporated under compressed air flow and the residue was purified by RP-HPLC, obtaining compound 14a as white foam. Yield 73% (0.012 g).

1H NMR (400 MHz, D2O) δ: 4.50 (t, J = 6.7 Hz, 1H, CH), 3.52 (t, J = 5.0 Hz, 2H, CH2N), 3.34-3.27 (m, 5H, OCH2, CH2O), 3.19 (t, J = 6.8 Hz, 2H, NCH2), 2.09-1.89 (m, 2H, CH2), 1.69-1.57 (m, 2H, CH2).

13C (100 MHz, D2O) δ: 156.3 (C Ar, CN3), 70.3 (OCH2), 46.5 (CH), 41.1 (NCH2), 40.2 (CH2N), 27.5 (CH2), 23.4 (CH3).


HRMS calcd. for C10H11ClN6O3: 311.1, found (relative intensity) 311.1 (100) [M+H]+.

HRMS calcd. for C8H8N5O2: 174.1, found (relative intensity) 174.1 (100) [M+H]+.

Triazoles synthesis

General azide synthesis method (Procedure F)

To a stirring solution of alcohol 15 or 22 (1 eq) in toluene (5 mL), diphenylphosphoryl azide (1.2 eq) and DBU (1.3 eq) were added. The reaction was stirred overnight at room temperature, diluted with water (10 mL) and extracted with AcOEt (3x10 mL). The combined organic phases were washed with brine (10 mL), dried over anhydrous sodium sulphate and the solvent evaporated. The residue was purified by flash column chromatography (20% AcOEt- n-Hexane) to achieve the desired azide 16 or 23 respectively as a colourless oils.

[Synthesis and complete characterization of compound 18 and 21 was reported by Gijsen, et al. (2013) and Zornik, D. et al. (2011), respectively.]

General triazole synthesis method. (Procedure G)

The alkyne 18 or 21 (1 eq) and the azide 16 or 23 (1 eq) were dissolved in DCM (10 mL). Copper iodide (0.2 eq) and triethylamine (1 eq) were added. The reaction was stirred for 24 hours at room temperature then a saturated solution of ammonium chloride was added (10 mL). After stirring for 5 minutes the mixture was extracted with DCM (3x10 mL), washed with brine (10 mL), dried over anhydrous sodium sulphate and the solvent was evaporated. The residue was purified by flash column chromatography (2% Methanol in Dichloromethane) to achieve the desired product 19 or 24 respectively as white foams.

2-(Azidomethyl)-1H-benzo[d]imidazole. 16

(Procedure F) Yield 83% (0.144 g), Rf 0.36 (n-Hexane/AcOEt 70:30).

1H NMR (400 MHz, CDCl3) δ: 11.76 (s, 1H, NH), 7.55 (dd, J = 6.0, 3.2 Hz, 2H, 2xArH), 7.21 (dd, J = 6.1, 3.1 Hz, 2H, 2xArH), 4.66 (s, 2H, CH2).

13C NMR (101 MHz, CDCl3) δ: 149.1 (2xCAr), 138.4 (C Ar), 123.2 (2xCAr), 115.4 (2xCAr), 48.4 (CH2).

ESI MS m/z: [M+H]+ calcd for C8H8N7 233.1, found (relative intensity) 233.1 (100) [M+H]+.

HRMS calcd. for C8H8N7O: 233.0631, found: 233.0629.

2-(4-Chloropyridin-2-yl)-1H-1,2,3-triazol-1-yl)methyl]-1H-benzo[d]imidazole. 19

(Procedure G) Yield 30% (0.092 g), Rf 0.27 (Dichloromethane/Methanol 95:5).

1H NMR (400 MHz, DMSO) δ: 12.68 (br, 1H, NH), 8.81 (s, 1H, CH), 8.61 (d, J = 5.3 Hz, 1H, ArH), 8.08 (d, J = 1.8 Hz, 1H, ArH), 7.59-7.50 (m, 3H, 3x ArH), 7.25-7.14 (m, 2H, 2x ArH), 5.98 (s, 2H, CH2).

13C NMR (100 MHz, DMSO) δ: 151.9 (2xCAr), 151.8 (C Ar), 148.7 (C Ar), 146.6 (C Ar), 144.3 (2xCAr), 125.4 (CH), 123.5 (3xCAr), 122.6 (2xCAr), 119.7 (C Ar), 48.1 (CH2).


HRMS calcd. for C15H12ClN6Na: 333.0631, found: 333.0629.

2-(Azidomethyl)-4-chloropyridine. 23

(Procedure F) Yield 55% (0.091 g), Rf 0.51 (n-Hexane/AcOEt 70:30).

1H NMR (400 MHz, CDC13) δ: 8.41 (d, J = 5.3 Hz, 1H, ArH), 7.30 (d, J = 1.6 Hz, 1H, ArH), 7.22 – 7.14 (m, 1H, ArH), 4.42 (s, 2H, CH2).
\[^{13}\text{C}\] NMR (100 MHz, CDCl\textsubscript{3}) \(\delta\): 157.5 (C\textsubscript{Ar}), 150.4 (C\textsubscript{Ar}), 144.9 (C\textsubscript{Ar}), 123.2 (C\textsubscript{Ar}), 122.1 (C\textsubscript{Ar}), 55.0 (CH\textsubscript{3}).

ESI MS \(m/z\): [M+H]\(^+\) calcd for C\textsubscript{16}H\textsubscript{12}C\textsubscript{4}N\textsubscript{4} 169.0, found (relative intensity)169.1 (100) [M+H]\(^+\).

2-[(4-Chloropyridin-2-yl)methyl]-1H-1,2,3-triazol-4-yl]-1H-benzo[d]imidazole. 24

(Procedure G) Yield 65% (0.198 g), \(R_f\) 0.31 (Dichloromethane/Methanol 95:5).

\[^{1}\text{H}\] NMR (400 MHz, DMSO) \(\delta\): 13.10 (br, 1H, NH), 8.86 (s, 1H, CH\textsubscript{3}), 8.54 (d, \(J = 5.3\) Hz, 1H, ArH), 7.69-7.60 (m, 2H, 2x ArH), 7.58 (dd, \(J = 5.3, 2.0\) Hz, 1H, ArH), 7.53-7.48 (m, 1H, ArH), 7.27-7.14 (m, 2H, 2x ArH), 5.89 (s, 2H, CH\textsubscript{3}).

\[^{13}\text{C}\] NMR (100 MHz, DMSO) \(\delta\): 157.0 (C\textsubscript{Ar}), 151.5 (C\textsubscript{Ar}), 144.9 (C\textsubscript{Ar}), 144.2 (C\textsubscript{Ar}), 144.1 (C\textsubscript{Ar}), 140.0 (C\textsubscript{Ar}), 134.8 (C\textsubscript{Ar}), 125.8 (C\textsubscript{Ar}), 124.0 (C\textsubscript{Ar}), 123.1 (C\textsubscript{Ar}), 123.0 (C\textsubscript{Ar}), 122.1 (C\textsubscript{Ar}), 119.2 (C\textsubscript{Ar}), 112.0 (C\textsubscript{Ar}), 54.4 (CH\textsubscript{3}).

ESI MS \(m/z\): [M+H]\(^+\) calcd for C\textsubscript{16}H\textsubscript{12}C\textsubscript{4}N\textsubscript{4}Na 333.1, found (relative intensity) 311.1 (100) [M+H]\(^+\), 333.1 (20) [M+Na]\(^+\).

HRMS calcd. for C\textsubscript{16}H\textsubscript{12}C\textsubscript{4}N\textsubscript{4}Na: 311.0806, found: 311.0810.

Molecular Biology

DDAH-1 cloning and expression

The hDDAH-1 coding sequence (CDS; NM\_012137) was shuttled into the pEF-IRE5 mammalian expression vector. Cells were transfected with the pEF-IRE5-DDAH-1 expression construct (4 \mu g) using Lipofectamine2000 in OptiMEM (Invitrogen, CA, USA). The stable expression of hDDAH-1 was achieved in the human embryonic kidney cell line, HEK293T, according to the published procedure of Lewis et al.\(^{54}\)

Western blot analysis

Denatured total protein from recombinant expressions were loaded onto 4-10% PAGE gels, separated (170 V), then rectilinearly transferred to Trans-Blot\textsuperscript{\textregistered} Transfer Medium pure nitrocellulose (Biorad; 0.45 \mu m; 90V). Immunodetection of hDDAH-1 protein was achieved by probing blots with Goat polyclonal anti-DDAH-1 primary antibody (Abcam\textsuperscript{\textregistered}, UK) (1:3000 dilution) followed by ImmunoPure\textsuperscript{\textregistered} Rabbit anti-Goat IgG used as the secondary antibody (H+L; peroxidase conjugated; Rockford, IL, USA) (1:4000 dilution). Immunoreactivity was detected using the BM Chemiluminescence Blotting Substrate (Roche Diagnostics GmbH, Mannheim Germany) and recorded digitally using the FUJIFILM LAS-400 image reader (version 2.0; FUJIFILM Life Science Corporation; Tokyo, Japan) (Figure 5).

Lysate and pellet fractions of HEK293T cells transfected with pEF-IRE5-DDAH-1 (see DDAH-1 cloning and expression) demonstrated immunoreactivity when probed with anti-DDAH-1 primary antibody in contrast to untransfected HEK293T cell lysate and pellet fractions. Colorburst\textsuperscript{\textregistered} (8-220 kDa; Sigma-Aldrich, Australia) was used as the molecular weight marker (MM).

Measurement of L-citrulline formation by hDDAH-1

L-Citrulline formation was determined in glass tubes at 37 °C in a total incubation volume of 0.1 mL. Incubation mixtures contained HEK293T cell lysate expressing recombinant hDDAH-1 (0.5 mg/mL), phosphate buffer (0.1 M, pH 7.4) and ADA (0, 10, 20, 35, 50, 75, 100, 150, 250, 375 and 500 \mu M). Deuterated L-citrulline (d6-L-citrulline, 5 mg/L) was used as the internal standard to correct for random and systemic errors during sample preparation, chromatography and detection. Following a 5 min pre-incubation step (37 °C), reactions were initiated by the addition of substrate (ADMA). Incubations were terminated after 60 min by the addition of 200 \mu L of ice-cold acetic acid 4% in methanol. Reaction mixtures were vortex mixed, cooled on ice for 10 minutes, then centrifuged (6000 x g for 10 min at 10 °C). An aliquot of the supernatant fraction (150 \mu L) was diluted into 350 \mu L of mobile phase (3:10 dilution) and an aliquot of 5 \mu L was injected onto the UPLC-MS and monitored according to the method described by Van Dyk et al. (2015).\(^{55}\) L-Citrulline and ADMA were separated from the matrix in a WATERS ACQUITY T3 HSS C18 analytical column using a Waters ACQUITY Ultra Performance LC\textsuperscript{\textregistered} (UPLC) system. The analyte was separated by gradient elution at a flow rate of 0.2 mL/min in a mobile phase comprising 10 mM ammonium formate pH 3.0 in 0.5% methanol (Mobile phase A) and 10 mM ammonium formate pH 3.0 in 90% methanol (Mobile phase B) with gradient elution at 0.2 mL/min over 5 minutes. A WATERS QTOF premier mass spectrometer was used to quantify L-citrulline formation in ESI+ V mode detecting the citrulline fragment with a m/z = 159.1. Unknown concentrations of L-citrulline were determined by comparison of the peak area to a calibration curve constructed in the concentration range 0.5 to 25 \mu M.

The linearity of L-citrulline formation over time (0-120 min) and over protein concentration (0-1 mg/mL) was confirmed in preliminary experiments (data not shown). A protein concentration of 0.5 mg/mL and incubation time of 60 min was used in all experiments and ensured initial rate conditions with substrate utilization <20%.
Calculation of ADMA kinetic parameters

The rates of L-citrulline formation versus substrate concentration (0–500 μM) were model fitted using the nonlinear curve fitting program EnzFitter (version 2.0.18.0: Biosoft, Cambridge, UK). Kinetic constants (Km, Vmax) for L-citrulline formation were derived from fitting the Michaelis-Menten equation to experimental data. Goodness of fit was assessed from the F statistic, 95% confidence intervals, \( r^2 \) value, and standard error of the parameter fit. Kinetic data are given as the mean of four separate experiments and derived \( K_m \) and \( V_{max} \) values of 103 μM and 258 pmol/min/mg, respectively (Figure 6).

![Figure 6](image)

**Figure 6.** (A) Rate of reaction (pmol/min/mg protein) versus [ADMA] plot of L-citrulline formation by hDDAH-1. (B) Eadie-Hofstee plot for L-citrulline formation induced by hDDAH-1 at different ADMA concentrations. Units of Velocity/ADMA are pmol/citrulline/μmol/min/mg. Points are experimentally determined values, whereas lines are from model-fitting.

Analysis of DDAH inhibition

All compounds synthesized here along with the known DDAH inhibitor L-257 were assessed for their ability to inhibit hDDAH-1 by detecting L-citrulline formation. Inhibition experiments separately comprised each novel compound and L-257 (0-1 mM), HEK293T cell lysate expressing recombinant hDDAH-1 (0.5 mg/mL), phosphate buffer (0.1 M, pH 7.4) and ADMA (100 μM) as the substrate. Following a 5 min pre-incubation step (37 °C), reactions were initiated by the addition of ADMA. Deuterated L-citrulline (d6-L-cit, 5 mg/L) was used as the internal standard. Reactions were terminated and prepared for analysis (see Measurement of citrulline formation by hDDAH-1). Each potential inhibitor was used at five concentrations (0, 0.1, 1, 10, 100, 1000 μM or 0, 0.1, 1, 10, 100, 500 μM in the case of 1,2,3-triazoles) for screening studies and five concentrations in the range 0-75 μM in kinetic studies. DMSO in a final concentration of 1% was used in the experiments involving the 1,2,3-triazole compounds in order to permit solubilization.

Data points referring to inhibition screening are the mean of duplicate measurements and kinetic data are represented as Eadie-Hofstee plots. The concentrations of compounds producing 50% of enzyme inhibition ([IC50] were calculated using GraphPad Prism 5.0 and these values were derived only for compounds with significant inhibition at 1 mM. Inhibition constants (K) were determined by fitting data to the equations for competitive, non-competitive, uncompetitive, and the mixed model of inhibition using Enzfitter. Goodness of fit was assessed from the F statistic, 95% confidence intervals, \( r^2 \) value, and standard error of the parameter fit.

In silico analyses

**Protein and inhibitor preparation**

The structure of hDDAH-1 pdb entry 2J1V[52] was used for all studies. The GROMOS force field treats aliphatic hydrogen atoms as united atoms together with the carbon atom to which they are attached. The coordinates of polar hydrogen atoms (bound to nitrogen, oxygen, or sulfur atoms) and aromatic hydrogen atoms were generated based on ideal geometries. In the chain A of pdb 2J1V, the first 7 N-terminal residues were not resolved in addition to residues 32, 33, 282-284. The coordinates for the missing residues 1-7 and 32, 33 were built using the ModLoop program.[53] The missing C-terminal residues (282-284) were not included in the model as they are distal to the active-site. The charges of the ionizable groups were chosen to correspond to a pH of 7, resulting into charge of -8 for the system of hDDAH-1, 8 Na+ ions were added to get a system with net charge of zero. In the presence of L-257 (or 10a) 7 Na+ were added to neutralize the system to compensate +1 charge of each ligand. Histidine residues were assigned the appropriate tautomeric configuration based on the local environment of these residues. The protein was placed at the center of a periodic cubic box, which was filled with ~9987 SPC water molecules. For the simulations in the presence of L-257 (or 10a), the initial configurations were obtained by overlaying with X-ray crystal structure of L-257 co-crystallized with DDAH-1.

**Molecular Dynamics Simulations**

All molecular dynamics (MD) simulations were performed under periodic conditions using the GROMACS package v. 4.5.4[59] in conjunction with the GROMOS 53A6 force field.[60] The initial structure of hDDAH-1 pdb entry 2J1V[52] was used for all studies. The topologies of the ligands (L-257 and 10a) were generated using the ‘Automated Topology Builder’ (ATB, http://combio.biosci.uq.edu.au/ATB/). All systems were
energy-minimized using the steepest descent method. Equilibration was then conducted in two phases, during which position restraints were placed on all heavy protein atoms. The first phase applied an NVT ensemble for 250 ps, using the Berendsen weak coupling method to maintain the temperature of the system at 298 K. The protein and solvent (including ions) were coupled separately. The second phase of equilibration applied an NPT ensemble for 250 ps, using Parrinello-Rahman barostat to maintain the pressure at 1 atm. Production MD was then conducted in the absence of any restraints, using the same NPT ensemble. The lengths of all bonds were constrained to ideal values using the P-LINCS algorithm with an integration time step of 2 fs. The neighbour list was cut off at 1.4 nm and updated every 5 time steps. All short-range non-bonded interactions were cut off at 1.4 nm, with dispersion correction applied to energy and pressure terms to account for the truncation of van der Waals interactions. Long-range electrostatic interactions were calculated with the smooth particle mesh Ewald method, using cubic-spline interpolation and a Fourier grid spacing of approximately 0.16 nm. All analyses were conducted using programs in the GROMACS package. Visualization was done using VMD program.

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References
46. US 7709481 B2, .


