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Synthesis of two epimers of pseudopaline.

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Supporting Information Placeholder

ABSTRACT: Pseudopaline is an opine metal chelator recently identified in *Pseudomonas aeruginosa*. This metallophore plays an important role in bacterial development during infections, providing a route for the acquirement of essential micronutrients (Zn^{2+} and Mn^{2+}) in metal scarce environments. We present here a straightforward synthetic approach for the synthesis of two epimers of pseudopaline and the attribution of their absolute configurations.

1. INTRODUCTION

Opines constitute a large family of highly polar molecules with different structures. A substantial part of these molecules belongs to the octopine (**1**) and nopaline (**2**) classes (fig. 1), characterized by the presence of a *N*-1-carboxylalkyl amino acid group.¹

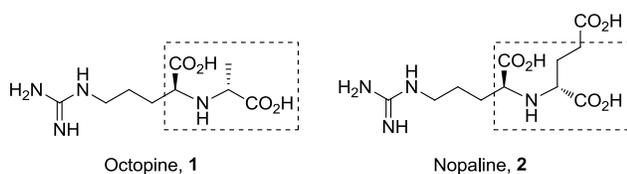


Figure 1. Structures of octopine (1) and nopaline (2). The characteristic moiety of opines is highlighted.

Such molecules were firstly identified in plant crown gall tumors, caused by *Agrobacterium spp.*,¹ and in lower marine invertebrate phyla and middle phyla of Protostomia.² Opines are synthesized by opine dehydrogenases (ODHs), which catalyse the reductive amination between different amino acids and ketoacids (often pyruvate, the dead-end product of glycolysis) while consuming nucleotinamide adenine dinucleotide (phosphate) (NAD(P)H).³ Different species of the genus *Agrobacterium* use opines as a selective advantage in their invasion of a variety of plants. These bacteria transfer their tumor-inducing (Ti) plasmid, which contains the genes encoding for opine synthesis, to plants cells. The latter transform in crown gall tumors and start producing opines which represent specific growth substrates for the bacteria; this is known as the “opine concept”.^{1,3-4} On the other hand, marine invertebrates (e.g. *Antarctica islandica* and *Asterina pectinifera*) synthesize opines in order to balance the cellular redox equilibrium. Similarly to the lactic acid fermentation, the synthesis of opines allows to re-oxidize NADH in hypoxic conditions, granting a continuous flux of glycolysis.^{2,5} Recently, two new opines, staphylopin (3)⁶ and pseudopaline (4),⁷ have been identified in *Staphylococcus aureus* and *Pseudomonas aeruginosa*, respectively. Compounds 3 and 4 have similar structures as depicted in figure 2: both molecules present a histidine (His) residue (with *R* or *S* configuration, respectively), a 2-aminobutyrate residue, and an end portion deriving from pyruvate (compound 3) or from α -ketoglutarate (compound 4). These two molecules are related to nicotianamine (compound 5, fig 2), an important plant siderophore.⁸ Compounds 3 and 4 do not participate in metabolism as the other opines, but are, like 5, soluble metallophores involved in a system which also comprehends the biosynthetic machinery and membrane reporters, whose function is to grant the supply of divalent metal cations to the bacteria.^{6,7b}

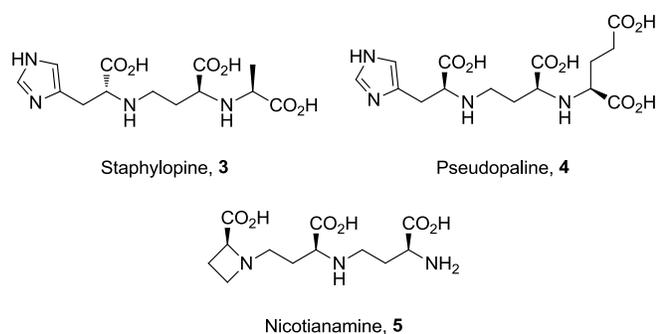


Figure 2. Structures of the three related metallophores staphylopin (3), pseudopaline (4) and nicotianamine (5).

In *P. aeruginosa*, pseudopaline is synthesized by two cytoplasmic enzymes encoded by the *cntL* and *cntM* elements of a four gene operon. The *cnt* operon also encodes the CntI and CntO proteins respectively involved in pseudopaline export and recovery by the bacteria.^{7b,9} *In vivo*, the pseudopaline operon is induced under zinc limitation in line with the in-

volvement of pseudopaline in zinc uptake in metal scarce conditions (it is also involved in manganese uptake).^{7b} The role of pseudopaline is crucial for *P. aeruginosa* growth in such metal scarce conditions recovered during infections where the nutritional immunity framework (basically the host-guest competition for micronutrients) renders particularly difficult the supplying of metals.^{7b, 9-10} The interest around this molecule is particularly relevant in the case of cystic fibrosis (CF): *P. aeruginosa* causes lung infections in the 60-70% of adult patients, leading to a progressive pulmonary insufficiency, the principal cause of death.¹¹ Given the impact of the Cnt systems in *P. aeruginosa* and *S. aureus* infections, knowledge on metallophores production and control of metal level are highly relevant. After our recent characterization of SaCntM (the ODH of *S. aureus*), and in particular its peculiar activity modification in presence of different divalent metals,¹² we focused on the *P. aeruginosa* case. The full understanding of this metal acquisition system might disclose the route to novel antimicrobial therapies that could improve the life expectation of CF or other immunocompromise patients.

Recently, Zhang et al. proposed a synthesis for **3** and **4**.¹³ Their strategy, similarly to the synthesis that we previously published for compound **5**,¹⁴ relies on the conversion of amines in sulfonamides, followed by *N*-alkylations for the construction of the main chain of the molecule. This approach implies drastic conditions for the final deprotection reaction (i.e. the use of triflic acid). To approach the synthesis of pseudopaline **4** we designed a synthesis that could afford both epimers of **4** (namely compounds **4a** and **4b**, fig. 3) as single enantiomers at one time, in a convenient way, both in terms of number of steps and overall yield.

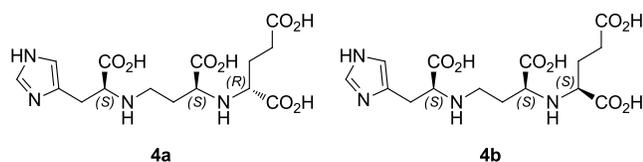
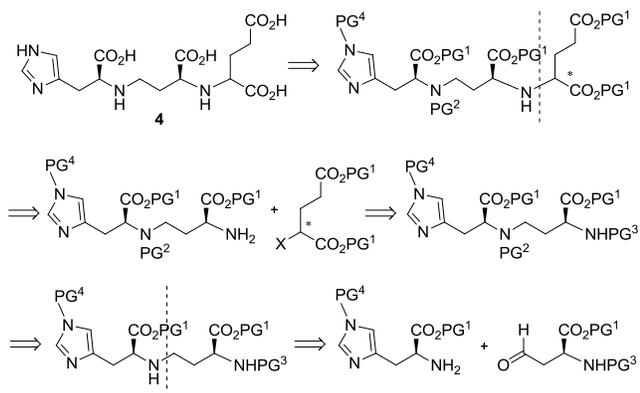


Figure 3. Structure of the two epimers of pseudopaline **4a** and **4b**.

2. RESULTS AND DISCUSSION

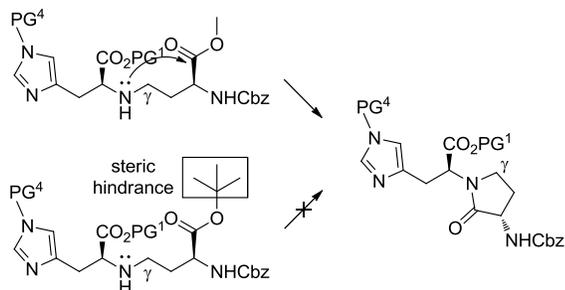
From a retrosynthetic point of view (scheme 1), **4** can be obtained from its protected version, ideally with all protecting groups with the same lability in order to facilitate the final deprotection. This intermediate can be disconnected in an α -haloglutarate ester and a primary amine. Its protection PG³ at the previous stage must have an orthogonal stability compared to the other protecting groups. This fully protected intermediate can derive from its corresponding unprotected secondary amine, which could be finally disconnected in a protected histidine and an aspartic semi-aldehyde analogue.

Scheme 1. Proposed retrosynthesis of **4**.



We planned to protect all carboxylic acids as *tert*-butyl ester for two practical reasons: *i*) they are easily cleaved under strong acidic condition resulting in a neat reaction with only volatile by-products and *ii*) the steric hindrance prevent that the secondary amine in position γ attacks the carbonyl to generate an undesired γ -lactam by intramolecular reaction (scheme 2).

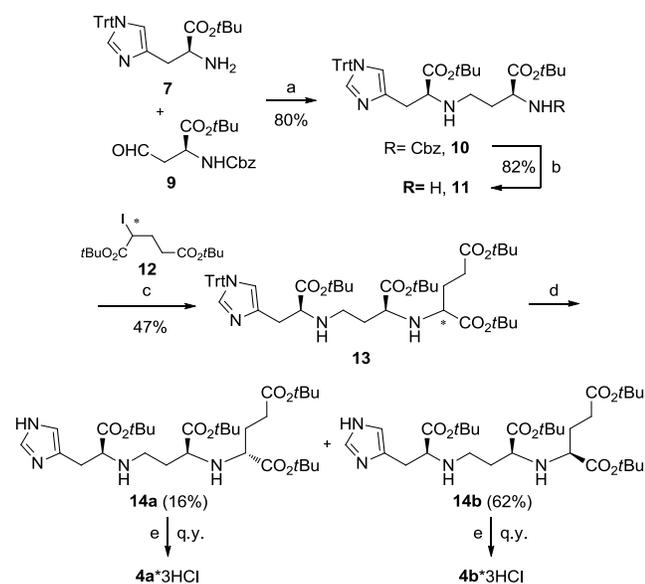
Scheme 2. Intramolecular side reaction.



In accordance to the *tert*-butyl ester protection strategy, trityl (Trt) has been chosen as protecting group for the N(τ) of His. Trt is more robust than other acid labile groups: for example, N(τ) Boc protection was not stable even in presence of weak bases (i.e. K_2CO_3 , $NaHCO_3$) at room temperature (r.t.).¹⁵ H-His(Trt)-OtBu (**7**), despite commercially available, was prepared in a three step procedure (overall yields 40-50%) starting from Cbz-His-OH (**6**, scheme S1). Following a similar reduction/selective oxidation strategy to the one that we recently reported,¹² we synthesized protected aspartic acid semi aldehyde (Asa), Cbz-Asa-OtBu (**9**) starting from Cbz-Asp-OtBu-dicyclohexylamine (DCHA) (**8**, scheme S2). Reductive amination between synthons **7** and **9** proceeded in good yields (80%, scheme 3) without the formation of any potential byproduct, such as tertiary amine deriving from a second reductive amination, or γ -lactam deriving from the intramolecular attack of the secondary amine to the ester group of **10**. Compound **10** was submitted to hydrogenolysis

affording **11** in good yields, which was engaged in alkylation reaction (scheme 3) with di-*tert*-butyl 2-iodoglutarate **12** (prepared starting from L-glutamic acid, scheme S3). The reactivity of **12** is somehow limited (probably for steric reasons), as alkylation proceeded in a regioselective fashion at the more accessible primary amine of **11**, as was verified by bi-dimensional nuclear magnetic resonance (NMR) analysis (see S.I.). After high performance liquid chromatography (HPLC) purification, compound **13** was obtained as an epimeric mixture in 47% yield. We also performed the same transformations starting from Boc protected **10** (scheme S5), invariably obtaining the same epimeric mixture, but in lower yields.

Scheme 3. Synthesis of **4a** and **4b**.



Reagents and conditions: a) i. anh. MgSO_4 , dry MeOH, r.t., 3h; ii sodium triacetoxylborohydride (STAB), r.t., 16h; b) H_2 (atm. press.), 10% Pd/C, *i*PrOH, r.t., 4 h; c) K_2CO_3 , dry *N,N*-dimethylformamide (DMF), 40°C; d) 20% $\text{HCO}_2\text{H}/\text{MeOH}$, cat. TIS, 40 °C, overnight (o.n.); e) 37% aq. HCl, r.t., 3h.

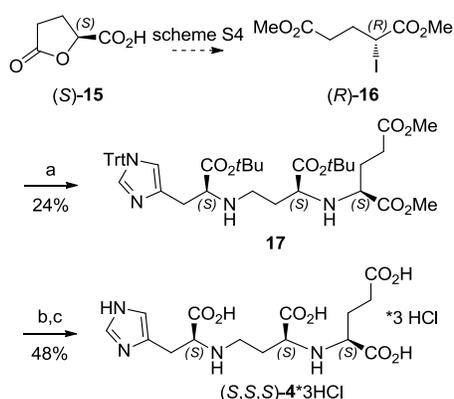
With the aim of isolating each epimers, compound **13** was treated with diluted formic acid in presence of triisopropylsilane (TIS) as scavenger to selectively remove the Trt group, leading to epimers **14a** and **14b** which were separated by silica gel column chromatography. However, **14a** required additional HPLC purification to increase its chemical purity (low preparative HPLC recovery is ascribed to the reduced yield for **14a**). Finally, treatment of both **14a** and **14b** with concentrated HCl afforded the two epimers of pseudopaline, **4a** (6 mg, $[\alpha]_{\text{D}}^{20} -9.1$ (c 0.55 g/dL, H_2O)) and **4b** (22 mg,

$[\alpha]_D^{20} +18.9$ (c 0.55 g/dL, H₂O)) in 24% overall yields (starting from the three building blocks, scheme 3). The two molecules however revealed instability upon standing at 0-4 °C forming lactams by condensations, as suggested both by MS analysis (the molecular ion peak has a loss of 18 in *m/z* compared from **4**, compatible with the loss of a water molecule) and NMR spectra (the signals of the α amino acidic protons moves at lower fields, compatible with the presence of a lactam, see S.I.). This undesired conversion is probably prompted by acidic conditions, so in future work, different deprotection condition should be considered. A possible alternative would be represented by the hydrolysis of the esters under basic conditions, which would afford **4** in its deprotonated form, unreactive to lactamisation.

Remarkably, the two epimers present ¹H NMR spectra (fig. 4) with peculiar signals, especially around 4.1 ppm. For the attribution of the absolute configuration of **4a** and **4b**, we needed enantiopure **4** with a known configuration. Starting from commercial lacton (*S*)-**15**, we synthesized enantiopure alkyl iodide (*R*)-**16** (scheme 4). The carboxylic acids were protected as methyl esters since attempts of obtaining the corresponding di-*tert*-butyl ester failed. (*R*)-**16** was reacted with **11** affording **17** (scheme 4), a fully protected equivalent of (*S,S,S*)-**4**.

The crude product presented an impurity with the same molecular mass of **17** (with a ratio 3:1 **17**/impurity, see S.I.). As a S_N1 mechanism is unlikely, we speculated that the presence of the less hindered methyl esters in (*R*)-**16**, compared to the *tert*-butyl esters in compound **12**, might allow alkylation of the secondary amine of **11**, resulting in the regioisomeric by-product. As confirmation, deprotection of the byproduct never afforded **4** (in any form). Compound **17** was deprotected by hydrolysis followed by acidic treatment, affording (*S,S,S*)-**4** (scheme 4). Comparison of the ¹H NMR spectra unambiguously showed the correspondence between (*S,S,S*)-**4** and **4b** (fig. 4); by consequence, we attributed the (*S,S,R*) configuration to **4a**.

Scheme 4. Synthesis of (*S,S,S*)-**4**



Reagents and conditions: a) **11**, K₂CO₃, dry DMF, 40°C; b) 1 M aq. LiOH, tetrahydrofuran (THF), 2h, r.t.; c) 37% aq. HCl, cat. TIS, r.t., 4 h.

By the time this article was prepared, Zhang et al.¹⁶ published the asymmetric synthesis of **4a** and **4b**, along with the attribution of the (*S,S,S*) configuration (the one of **4b**) to the natural product. This synthesis relies, as the one the same authors proposed for compound **3**,¹³ on the conversion of the amino groups in sulfonamides which are engaged, in turn, in an asymmetric Tsuji-Trost and a Fukuyama-Mitsunobu reactions. This elegant strategy presents anyhow some drawbacks, as it requires harsh deprotection conditions and a series of oxidations involving highly toxic reagents (i.e. OsO₄, Jones Reagent).

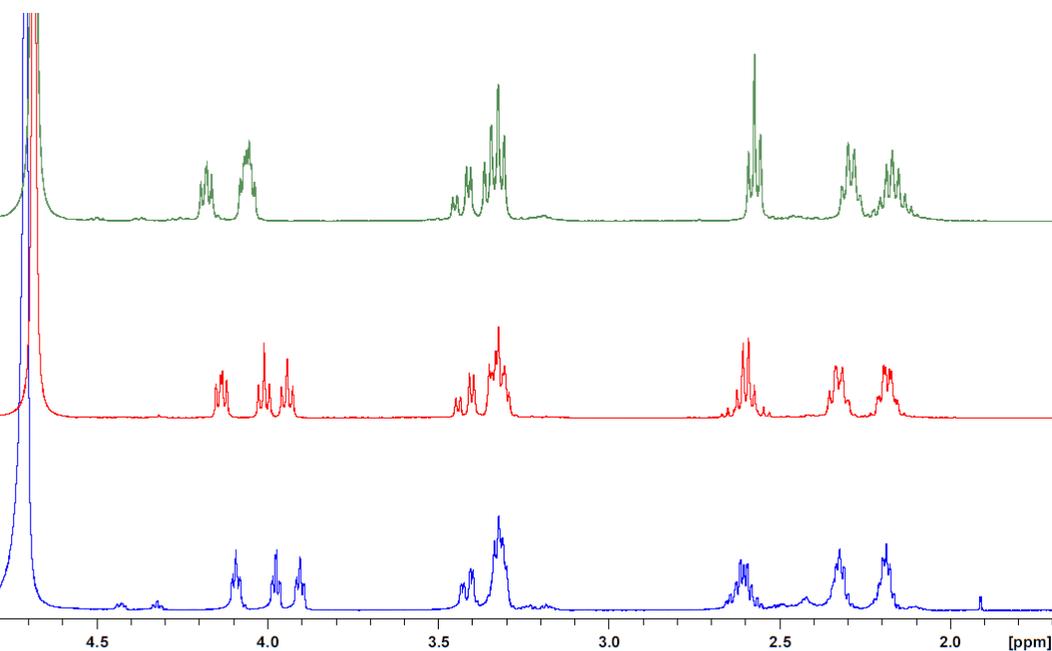


Figure 4. ¹H NMR (D₂O) comparison. The spectra of (*S,S,S*)-**4** (registered at 600 MHz, blue) does not correspond to the one of **4a** (400 MHz, green), but it does to the one of **4b** (400 MHz, red). The little difference in the δ of H₂O signals (≤ 0.2 ppm) is within acceptable limits, considering the different condition of the acquisitions.

3. CONCLUSIONS

In this paper, we present a new synthesis of the two epimers of pseudopaline (**4**). This approach represents an alternative strategy for the synthesis of pseudopaline (and, in general, of opine metallophores) to the one exploited by Zhang et al.,¹⁶ thanks to a reduced number of steps (5, starting from commercially available **7**, vs 8), comparable overall yields (24% vs 22%), and more green conditions avoiding the use of transition metals, toxic oxidating reagents, and of non-atom efficient reactions (i.e. Fukuyama-Mitsunobu). Epimers **4a** and **4b**, differing at the absolute configuration of the glutarate portion, were obtained as single enantiomers after resolution by column chromatography at the n-1 step of the synthesis. Starting from chiral building block (*S*)-**15** we synthesized (*S,S,S*)-**4**, which allowed us to assign the absolute configuration of **4a** and **4b**. This second approach is less efficient than the first synthetic strategy (8% vs 24%), due to a side reaction in the alkylation step. A different protection for the diester (*R*)-**16** (with larger groups), or the protection of the secondary amine of **10** before the hydrogenolysis of the Cbz (benzyloxycarbonyl) group, might avoid this problem. Our approach has different advantages, as a reduced number of steps, scale-up easiness (the main problem might be the purification of intermediates **14a** and **14b**) and flexibility (using different building blocks it is possible to obtain different analogues of **4**). This strategy appears thus to us suitable for an eventual drug discovery campaign and for chelation studies of pseudopaline with different metals.

4. EXPERIMENTAL SECTION

General procedures

Synthesis and characterization of compounds **7**, **9**, **12**, and (*R*)-**16** are reported in the supporting informations. All reagents and solvents were purchased from commercial suppliers (Merck, Fluorochem, TCI Europe, Carlo Erba, IRIS biothec, Fluka and AlfaAesar). All reaction involving air-sensitive reagents were performed under argon atmosphere. Catalytic hydrogenations were performed at atmospheric pressure. Solvent ratios are intended as a v/v ratios. Reaction were checked by TLC on commercial silica gel 60 F254 aluminum sheets (spots were further evidenced by spraying with a dilute alkaline solution of KMnO₄) or by RP-Analytic HPLC performed on an Agilent 1220 using a 50 x 4.6 mm Chromolith® High Resolution column. Compounds were separated using solvent A (H₂O + 0.1 % TFA) and solvent B (MeCN + 0.1 % TFA) with a linear gradient of solvent B from 0 to 100% in 3 min using a constant flow rate of 3 mL min⁻¹. Purifications were performed with column chromatography using silica gel (Merck 60, 230–400 mesh) or by preparative

HPLC performed on a Gilson PLC2020 apparatus equipped with a Phenomenex Luna 5 μm C18(2) 100 \AA , 75 x 21.2 cm column coupled to an UV detector (detection fixed at 215 and 245 nm). Solvent A (H_2O + 0.1 % HCO_2H) and solvent B (MeCN + 0.1 % HCO_2H) were used with a constant gradient of solvent B from 0% to 100% in 12.8 min and a constant flow rate of 25.5 mL min^{-1} . Fractions of interest were collected, neutralized with solid NaHCO_3 and volume was reduced under reduced pressure. Product was extracted with DCM, pooled organic phase was dried over anhydrous Na_2SO_4 , filtered and solvent removed under reduced pressure obtaining the desired product. Nuclear magnetic resonance spectra were recorded on a Bruker spectrometer Avance 300 (300 MHz), on a Bruker Avance III spectrometer 400 (400 MHz), or on a Bruker Avance III spectrometer 600 (600 MHz). Chemical shifts (δ) are reported from tetramethylsilane with the solvent resonance as the internal standard (according to literature data¹⁷). Data are reported as follows: chemical shift (δ in ppm), multiplicity (s= singlet, d= doublet, t= triplet, b= broad, m= multiplet), coupling constants (J in Hz), integration, attribution. Low resolution electrospray ionization (ESI) mass spectra were recorded on a micromass platform electrospray mass spectrometer. Spectra were recorded in the positive mode (ESI+) or negative mode (ESI-, when specified). LC-MS system consisted of a Waters Alliance 2690 HPLC, coupled to a ZQ spectrometer (Manchester, UK), fitted with an electrospray source operated in the positive (ESI+) or negative (ESI-, when specified) ionization mode. All the analyses were carried out using a C18 Chromolith Flash 25 mm \times 4.6 mm column operated at a flow rate of 3 mL/min . A gradient of 0% or 0.1% aqueous TFA (solvent A) to 100% of acetonitrile containing 0.1% TFA (solvent B) was developed over 3 min. Positive-ion electrospray mass spectra were acquired at a solvent flow rate of 100–200 $\mu\text{L/min}$. Nitrogen was used for both the nebulizing and drying gas. Rotary power determinations were carried out using a PerkinElmer Polarimeter 341; reported concentrations have the dimensions of g/dL. High-resolution mass spectra (HRMS) were performed by the “Laboratoire de Mesures Physiques” of Montpellier University on a Micromass Q-ToF spectrometer equipped with electrospray source ionization (ESI), using phosphoric acid as internal standard.

(*S*)-*tert*-Butyl 2-(((benzyloxy)carbonyl)amino)-4-(((*S*)-1-(*tert*-butoxy)-1-oxo-3-(1-trityl-1*H*-imidazol-4-yl)propan-2-yl)amino)butanoate (**10**) Amine **7** (300 mg, 0.661 mmol) and aldehyde **9** (300 mg, 0.661 mmol) were dissolved in dry MeOH. Anhydrous MgSO_4 (120 mg) was added and the mixture was stirred at r.t. for 1 h. STAB (420 mg, 1.98 mmol) was added in small portions over 1 h. The reaction mixture was stirred at r.t. o.n., then it was filtered over a celite pad and the solvent was removed under reduced pressure. The residue was dissolved in DCM and washed with sat. aq. NaHCO_3 , dried over anhydrous Na_2SO_4 , filtered and the solvent was removed under reduced pressure. The crude was purified by column chromatography on SiO_2 gel with 3:7 $\text{CH}_2\text{Cl}_2/\text{EtOAc}$ + 2% of 28% aq. NH_3 affording the title compound as a white foam (393 mg, 80% yield). $R_f = 0.38$ (3:7 $\text{CH}_2\text{Cl}_2/\text{EtOAc}$ + 2 drops of 28% aq. NH_3). $^1\text{H NMR}$ (300 MHz, CDCl_3): δ 7.34 (s, 1H), 7.33-7.27

(m, 14H), 7.17-7.04 (m, 6H), 6.59 (s, 1H), 6.25 (d, $J = 7.7$ Hz, 1H), 5.05 (s, 2H), 4.26 (ddd, $J = 5.5, 7.3, 7.7$ Hz, 1H), 3.42 (dd, $J = 6.2, 6.4$ Hz, 1H), 2.89 (dd, $J = 6.2, 14.5$ Hz, 1H), 2.83-2.67 (m, 2H), 2.60 (m, 1H), 2.07 (bs, 1H), 1.98-1.73 (m, 2H), 1.43 (s, 9H), 1.35 (s, 9H). ^{13}C NMR (75 MHz, CDCl_3): δ 173.5, 171.6, 156.2, 142.6, 138.6, 137.3, 136.7, 129.9, 128.5, 128.1, 119.2, 81.7, 80.9, 75.2, 66.7, 61.9, 53.6, 44.1, 32.1, 31.9, 28.2, 28.1. MS(ESI): m/z calcd for $\text{C}_{45}\text{H}_{52}\text{N}_4\text{O}_6$: 744.4; found: 745.2(38) $[\text{M}+\text{H}]^+$, 503.2(85) $[\text{M}-\text{Trt}+\text{H}]^+$, 447.1(5) $[\text{M}-\text{Trt}-t\text{Bu}+\text{H}]^+$, 243.1(100) $[\text{Trt}]^+$. $[\alpha]_D^{20}$ -0.95 (c 1.16, CHCl_3).

(S)-tert-Butyl 2-amino-4-(((S)-1-(tert-butoxy)-1-oxo-3-(1-trityl-1H-imidazol-4-yl)propan-2-yl)amino)butanoate (11) Compound **10** (154 mg, 0.207 mmol) was dissolved in *i*PrOH (2.5 mL) in a sealed round bottom flask under argon. 10% Pd/C (36 mg) was added and the reaction mixture was stirred at r.t. under hydrogen for 4 h. The mixture was filtered over a celite pad then the solvent was removed under reduced pressure. The crude was purified by column chromatography on SiO_2 gel with affording the title compound as a pale yellow oil (103 mg, 82% yield). $R_f = 0.27$ (95:5 DCM/MeOH + 2 drops of 28% aq NH_3). ^1H NMR (400 MHz, CDCl_3): δ 7.31 (s, 1H), 7.31-7.26 (m, 9H), 7.15-7.05 (m, 6H), 6.60 (s, 1H), 3.43 (dd, $J = 5.8, 7.0$ Hz, 1H), 3.34 (dd, $J = 4.5, 8.3$ Hz, 1H), 2.90 (dd, $J = 5.8, 14.4$ Hz, 1H), 2.82-2.69 (m, 2H), 2.59 (m, 1H), 1.84 (m, 1H), 1.71 (bs, 3H), 1.57 (m, 1H), 1.42 (s, 9H), 1.36 (s, 9H). ^{13}C NMR (101 MHz, CDCl_3): δ 175.3, 173.8, 142.6, 138.5, 137.5, 129.8, 128.0, 119.3, 80.8, 80.8, 75.1, 61.9, 53.7, 44.9, 34.9, 32.3, 28.1, 28.2, 28.1. MS(ESI): m/z calcd for $\text{C}_{37}\text{H}_{46}\text{N}_4\text{O}_4$: 610.3; found: 611.3(38) $[\text{M}+\text{H}]^+$, 369.2(60) $[\text{M}-\text{Trt}+\text{H}]^+$, 243.1(100) $[\text{Trt}]^+$. $[\alpha]_D^{20}$ +2.7 (c 1.04, CHCl_3).

Di-tert-butyl 2-(((S)-1-(tert-butoxy)-4-(((S)-1-(tert-butoxy)-1-oxo-3-(1-trityl-1H-imidazol-4-yl)propan-2-yl)amino)-1-oxobutan-2-yl)amino)pentanedioate (13) Amine **11** (342 mg, 0.560 mmol) was dissolved in dry DMF (1 mL). K_2CO_3 (101 mg, 0.728 mmol) and a solution of iodide **12** (269 mg, 0.728 mmol) in dry DMF (1.8 mL) were added. The reaction was stirred at 45°C until complete conversion. Water was added and extracted twice with EtOAc. The pooled organic phase was dried over anhydrous Na_2SO_4 , filtered and the solvent was removed under reduced pressure. The crude was firstly purified by column chromatography on SiO_2 gel with 96:4 DCM/MeOH + 2% of 28% aq. NH_3 and the obtained compound (344 mg) was further purified by prep. HPLC (see general procedures). The product was obtained as a 1:1 mixture of epimers as a pale yellow oil (245 mg, 47% yield). $R_f = 0.39$ and 0.29 (95:5 DCM/MeOH + 2 drops of 28% aq. NH_3). ^1H NMR (400 MHz, CDCl_3): 1:1 mix of epimers δ 7.36-7.27 (m, 10H), 7.15-7.07 (m, 6H), 6.60 (s, 1H), 3.48-3.36 (m, 1H), 3.19-3.03 (m, 2H), 2.89 (dd, $J = 6.3, 14.4$ Hz, 1H), 2.79 (dd, $J = 6.3, 14.4$ Hz, 1H), 2.70 (m, 1H), 2.58 (m, 1H), 2.36-2.26 (m, 2H), 2.15 (bs, 2H), 1.94-1.82 (m, 1H), 1.82-1.55 (m, 3H), 1.48-1.38 (m, 27H), 1.38-1.31 (m, 9H). ^{13}C NMR (101 MHz, CDCl_3): 1:1 mix of epimers δ 173.9, 173.7, 172.8, 172.5, 142.7, 138.5, 137.5 and 137.5, 129.9, 128.1 and 128.1, 119.3 and 119.3, 81.2, 81.2, 81.1, 81.0, 80.7, 80.2, 80.2, 75.2, 62.2 and 62.1, 59.8 and 59.8, 59.3 and 59.2, 45.1 and 44.7, 34.1, 32.5, 32.0 and 31.8, 28.9 and 28.8, 28.2, 28.2. MS(ESI): m/z calcd for $\text{C}_{50}\text{H}_{68}\text{N}_4\text{O}_8$: 852.5; found: 853.4(30) $[\text{M}+\text{H}]^+$, 611.4(65) $[\text{M}-\text{Trt}+\text{H}]^+$, 243.1(100) $[\text{Trt}]^+$.

Synthesis of epimers **14a** and **14b**

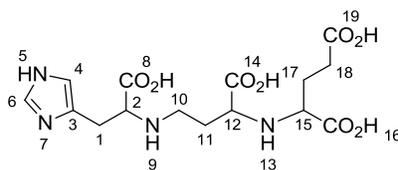
The mixture of **13** (110 mg, 0.129 mmol) was dissolved in DCM (0.8 mL). TIS (39.4 μ L, 0.195 mmol) and formic acid (0.2 mL) were added and the mixture was stirred at 40 °C o.n. Reaction was diluted with DCM, sat. aq. NaHCO₃ was added, phases were separated and the aqueous one was extracted with DCM. The pooled organic phase was dried over anhydrous Na₂SO₄, filtered and the solvent was removed under reduced pressure. The crude was purified by column chromatography on SiO₂ gel with 95:5 DCM/MeOH + 2% of 28% aq. NH₃ affording a fraction containing **14a** and pure **14b** (49 mg, 62% yield). **14a** was further purified by prep HPLC (see general procedures) affording pure **14a** (13 mg, 16% yield). The products were obtained as colourless oils.

(R)-Di-tert-butyl 2-(((S)-1-(tert-butoxy)-4-(((S)-1-(tert-butoxy)-3-(1H-imidazol-4-yl)-1-oxopropan-2-yl)amino)-1-oxobutan-2-yl)amino)pentanedioate (14a**)** $R_f = 0.58$ (92:8 DCM/MeOH + 2 drops of 28% aq. NH₃). ¹H NMR (400 MHz, CDCl₃): δ 7.55 (s, 1H), 6.80 (s, 1H), 3.30-3.21 (m, 2H), 3.13 (dd, $J = 5.5, 7.4$ Hz, 1H), 2.93 (dd, $J = 3.3, 15.1$ Hz, 1H), 2.77-2.59 (m, 3H), 2.41-2.23 (m, 2H), 1.97-1.72 (m, 4H), 1.46 (s, 9H), 1.44 (s, 9H), 1.43 (s, 9H), 1.43 (s, 9H). ¹³C NMR (101 MHz, CDCl₃): δ 174.2, 173.7, 173.3, 172.8, 135.0, 81.8, 81.7, 81.5, 80.5, 62.2, 59.3, 58.1, 44.4, 32.2, 31.8, 28.7, 28.2, 28.2, 28.2, 28.2. MS(ESI): m/z calcd for C₃₁H₅₄N₄O₈: 610.4; found: 611.4(100) [M+H]⁺, 555.3(42) [M-*t*Bu+H]⁺, 499.3(65) [M-2*t*Bu+H]⁺, 443.1(72) [M-3*t*Bu+H]⁺, 387.1(32) [M-4*t*Bu+H]⁺.

(S)-Di-tert-butyl 2-(((S)-1-(tert-butoxy)-4-(((S)-1-(tert-butoxy)-3-(1H-imidazol-4-yl)-1-oxopropan-2-yl)amino)-1-oxobutan-2-yl)amino)pentanedioate (14b**)** $R_f = 0.53$ (92:8 DCM/MeOH + 2 drops of 28% aq. NH₃). ¹H NMR (400 MHz, CDCl₃): δ 7.55 (s, 1H), 6.80 (s, 1H), 3.32-3.26 (m, 2H), 3.17 (dd, $J = 6.1, 7.3$ Hz, 1H), 2.94 (dd, $J = 3.6, 15.1$ Hz, 1H), 2.78 (m, 1H), 2.75 (dd, $J = 8.2, 15.1$ Hz, 1H), 2.60 (ddd, $J = 5.7, 8.2, 11.2$ Hz, 1H), 2.35 (t, $J = 7.6$ Hz, 2H), 1.96-1.73 (m, 4H), 1.45 (s, 9H), 1.44 (s, 9H), 1.43 (s, 9H), 1.42 (s, 9H). ¹³C NMR (101 MHz, CDCl₃): δ 173.9, 173.9, 173.2, 172.6, 135.1, 81.8, 81.5, 81.5, 80.5, 62.2, 59.9, 59.2, 45.1, 33.4, 32.0, 28.8, 28.2, 28.1. MS(ESI): m/z calcd for C₃₁H₅₄N₄O₈: 610.4; found: 611.6(27) [M+H]⁺, 555.3(5) [M-*t*Bu+H]⁺, 499.4(10) [M-2*t*Bu+H]⁺, 443.3(6) [M-3*t*Bu+H]⁺.

General procedure for the synthesis of **4a** and **4b**

Compound **14a** or **14b** was dissolved in 37% aq. HCl (1.5 mL) and the reaction was stirred at r.t. for 4 h. The solution was freeze-dried affording the title compound as a white powder in quantitative yields (**4a**: 6 mg; **4b**: 22 mg).



(R)-2-(((S)-1-carboxy-3-(((S)-1-carboxy-2-(1H-imidazol-4-yl)ethyl)amino)propyl)amino) pentanedioic acid trihydrochloride (4a*3HCl) ¹H NMR (400 MHz, D₂O): δ 8.69 (d, *J* = 1.2 Hz, 1H), 7.45 (s, 1H, *H*₄), 4.28 (dd, *J* = 5.2, 7.3 Hz, 1H, *H*₂), 4.20-4.13 (m, 2H, *H*₁₅+*H*₁₂), 3.54 (dd, *J* = 5.2, 15.9 Hz, 1H, *H*₁), 3.49-3.39 (m, 3H, *H*'₁+*H*₁₀+*H*'₁₀), 2.68 (t, *J* = 7.0 Hz, 2H, *H*₁₈+*H*'₁₈), 2.40 (dt, *J* = 6.8, 7.4 Hz, 2H, *H*₁₁+*H*'₁₁), 2.27 (m, 2H, *H*₁₇+*H*'₁₇). ¹³C NMR (101 MHz, D₂O): δ 179.7 (*C*₁₉), 176.4 (*C*₁₆), 172.4 (*C*₁₄), 169.9 (*C*₈), 134.2 (*C*₆), 126.2 (*C*₃), 118.2 (*C*₄), 60.5 (*C*₂), 59.2 (*C*₁₅), 53.9 (*C*₁₂), 44.0 (*C*₁₀), 29.0 (*C*₁₈), 25.5 (*C*₁₁), 24.4 (*C*₁), 23.7 (*C*₁₇). MS(ESI): *m/z* calcd for C₁₅H₂₂N₄O₈: 386.1; found: 387.1(30) [M+H]⁺, 369.1(95) [M-18+H]⁺, 351.0(98) [M-36+H]⁺, 153.2(100). [α]_D²⁰ -9.1 (c 0.55, H₂O). HRMS(ES⁺): *m/z* calcd for C₁₅H₂₃N₄O₈: 387.1516; found: 387.1517 [M+H]⁺ (Δ = 0.3 ppm).

(S)-2-(((S)-1-carboxy-3-(((S)-1-carboxy-2-(1H-imidazol-4-yl)ethyl)amino)propyl)amino) pentanedioic acid trihydrochloride (4b*3HCl) ¹H NMR (400 MHz, D₂O): δ 8.67 (d, *J* = 1.4 Hz, 1H, *H*₆), 7.43 (s, 1H, *H*₄), 4.30 (dd, *J* = 5.2, 7.5 Hz, 1H, *H*₂), 4.16 (t, *J* = 6.5 Hz, 1H, *H*₁₅), 4.11 (t, *J* = 6.5 Hz, 1H, *H*₁₂), 3.52 (dd, *J* = 5.2, 15.9 Hz, 1H, *H*₁), 3.48-3.39 (m, 3H, *H*'₁+*H*₁₀+*H*'₁₀), 2.69 (dd, *J* = 7.1, 13.2 Hz, 2H, *H*₁₈+*H*'₁₈), 2.43 (m, 2H, *H*₁₁+*H*'₁₁), 2.29 (dd, *J* = 3.7, 7.0 Hz, 1H, *H*₁₇), 2.26 (dd, *J* = 4.1, 7.0 Hz, 1H, *H*'₁₇). ¹³C NMR (101 MHz, D₂O): δ 176.4 (*C*₁₉), 170.9 (*C*₁₆), 170.3 (*C*₁₄), 169.8 (*C*₈), 134.2 (*C*₆), 126.1 (*C*₃), 118.2 (*C*₄), 59.9 (*C*₁₅), 59.4 (*C*₂), 58.2 (*C*₁₂), 43.6 (*C*₁₀), 29.5 (*C*₁₈), 26.3 (*C*₁₁), 24.7 (*C*₁₇), 24.4 (*C*₁). MS(ESI): *m/z* calcd for C₁₅H₂₂N₄O₈: 386.1; found: 387.1(67) [M+H]⁺, 369.1(83) [M-18+H]⁺, 351.0(100) [M-36+H]⁺. [α]_D²⁰ +18.9 (c 0.55, H₂O). HRMS(ES⁺): *m/z* calcd for C₁₅H₂₃N₄O₈: 387.1516; found: 387.1517 [M+H]⁺ (Δ = 0.3 ppm).

(S)-Dimethyl 2-(((S)-1-(tert-butoxy)-4-(((S)-1-(tert-butoxy)-1-oxo-3-(1-trityl-1H-imidazol-4-yl)propan-2-yl)amino)-1-oxobutan-2-yl)amino)pentanedioate (17) Amine **11** (255 mg, 0.417 mmol) was dissolved in dry DMF (1 mL). NaHCO₃ (42 mg, 0.500 mmol) and a solution of iodure (*R*)-**16** (143 mg, 0.500 mmol) in dry DMF (1 mL) were added. The reaction was stirred at 45 °C until complete conversion. Water was added and extracted twice with EtOAc. The pooled organic phase was dried over anhydrous Na₂SO₄, filtered and the solvent was removed under reduced pressure. Purification column chromatography on SiO₂ gel with 98:2 to 95:5 DCM/MeOH + 2 drops of 28% aq. NH₃ afforded pure title compound as a yellow oil (76 mg, 24% yield). *R*_f = 0.32 (96:4 DCM/MeOH + 2 drops of 28% aq. NH₃). ¹H NMR (400 MHz, CDCl₃): δ 7.35-7.28 (m, 10H), 7.14-7.08 (m, 6H), 6.60 (d, *J* = 1.1 Hz, 1H), 3.67 (s, 3H), 3.62(s, 3H), 3.44 (m, 1H), 3.22 (dd, *J* = 5.5, 7.1 Hz, 1H), 3.13 (dd, *J* = 5.0, 7.7 Hz, 1H), 2.90 (dd, *J* = 6.4, 14.5 Hz, 1H), 2.80 (dd, *J* = 6.4, 14.5 Hz, 1H), 2.76-2.66 (m, 1H), 2.65-2.54 (m, 1H), 2.48-2.32 (m, 2H) 2.10 (bs, 2H), 2.01-1.81 (m, 2H), 1.80-1.68 (m, 1H), 1.68-1.57 (m, 1H), 1.42 (s, 9H), 1.36 (s, 9H). ¹³C NMR (101 MHz, CDCl₃): δ 174.5, 173.8, 173.7, 142.5, 138.4, 137.2, 129.8, 128.0, 119.3, 81.3, 81.0, 75.2, 61.8, 59.2, 59.1, 51.9, 51.6, 44.6, 33.8, 32.0, 30.1, 28.1, 28.1. MS(ESI): *m/z* calcd for C₄₄H₅₆N₄O₈: 768.4; found: 769.5(30) [M+H]⁺, 527.4(100) [M-Trt+H]⁺.

(S)-2-(((S)-1-Carboxy-3-(((S)-1-carboxy-2-(1H-imidazol-4-yl)ethyl)amino)propyl)amino)pentanedioic acid trihydrochloride [(S,S,S)-4*3HCl] Compound **17** (49 mg, 63.7 μ mol) was dissolved in THF (0.14 mL) and 1 M aq. LiOH (0.14 mL) was added. After stirring at room temperature for 2 h, the crude was purified by HPLC. The obtained product was dissolved in 37% aq HCl 0.5 mL). TIS (2 drops) was added and the reaction was stirred at r.t. for 4 h. The solution was freeze-dried affording the title compound as a white powder (15 mg, 48% yield). ¹H-NMR (600 MHz, D₂O): δ 8.68 (m, 1H, H₆), 7.43 (s, 1H, H₄), 4.17 (m, 1H, H₂), 4.06 (m, 1H, H₁₅), 3.99 (m, 1H, H₁₂), 3.50 (dd, *J* = 5.1, 15.7 Hz, 1H, H₁), 3.46-3.36 (m, 3H, H'₁+H'₁₀+H'₁₀), 2.75-2.63 (m, 2H, H₁₈+H'₁₈), 2.41 (m, 2H, H₁₁+H'₁₁), 2.27 (m, 2H, H₁₇+H'₁₇). ¹³C-NMR (151 MHz, D₂O): δ 176.5 (C₁₉), 171.4 (C₁₆), 171.0 (C₁₄), 170.4 (C₈), 134.1 (C₆), 126.4 (C₃), 118.0 (C₄), 60.5 (C₁₅), 59.9 (C₂), 59.0 (C₁₂), 43.8 (C₁₀), 29.6 (C₁₈), 26.4 (C₁₁), 24.9 (C₁₇), 24.6 (C₁). MS(ESI): *m/z* calcd for C₁₅H₂₂N₄O₈: 386.1; found: 386.9(100) [M+H]⁺, 369.1(60) [M-18+H]⁺, 351.0(82) [M-36+H]⁺. This compound corresponds to **4b**.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Synthetic procedures and characterization of compounds (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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