Bacterial sliding clamp inhibitors that mimic the sequential binding mechanism of endogenous linear motifs

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Publication Details

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Abstract
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Bacterial Sliding Clamp Inhibitors that Mimic the Sequential Binding Mechanism of Endogenous Linear Motifs

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ABSTRACT: The bacterial DNA replication machinery presents new targets for the development of antibiotics acting via novel mechanisms. One such target is the protein–protein interaction between the DNA sliding clamp and the conserved peptide linear motifs in DNA polymerases. We previously established that binding of linear motifs to the Escherichia coli sliding clamp occurs via a sequential mechanism that involves two subsites (I and II). Here, we report the development of small-molecule inhibitors that mimic this mechanism. The compounds contain tetrahydrocarbazole moieties as "anchors" to occupy subsite I. Functional groups appended at the tetrahydrocarbazole nitrogen bind to a channel gated by the sidechain of M362 and lie at the edge of subsite II. One derivative induced the formation of a new binding pocket, termed subsite III, by rearrangement of a
loop adjacent to subsite I. Discovery of the extended binding area will guide further inhibitor development.

INTRODUCTION

New classes of antibiotics acting via novel mechanisms are urgently needed to combat increasing antibiotic resistance.\textsuperscript{1-4} A rich source of potential new antibiotic targets is the bacterial DNA replisome.\textsuperscript{5} During bacterial DNA replication and repair, the β-sliding clamp subunit (SC) of the DNA polymerase III (Pol III) holoenzyme, a ring-shaped homodimeric protein, encircles DNA and confers high processivity to DNA synthesis (Figure 1A).\textsuperscript{6} Components of Pol III that interact with the SC at the same binding site include the α polymerase and ε proofreading subunits,\textsuperscript{7} as well as the clamp loader (γ complex).\textsuperscript{6} The variety of SC-focused interactions, the moderate number of SCs per cell (300–600 dimers),\textsuperscript{8} the SC’s conserved structure across bacterial species\textsuperscript{9-11} and its structural divergence from the equivalent human protein (proliferating cell nuclear antigen, PCNA)\textsuperscript{12, 13} make it an attractive target for antibiotic development.\textsuperscript{5, 14}

Proteins that bind to the SC use short linear motifs (LMs, consensus sequence QL\textsubscript{x1}L\textsubscript{x2}F/L: S/D preferred at x\textsubscript{1}, x\textsubscript{2} may be absent)\textsuperscript{15, 16} to interact with a LM-binding pocket consisting of two subsites (I and II),\textsuperscript{17, 18} both of which are conserved across Gram-negative and Gram-positive species.\textsuperscript{10} The two N-terminal residues of LMs bind in subsite II, while the two or three C-terminal residues occupy subsite I, with the linking residues filling a shallow channel between the sites. The side chain of SC residue M362
acts as a gate, transitioning from a “closed” (Figure 1B) to an “open” conformation (Figure 1C–E) upon binding of LM peptides.

We used structural, biochemical and computational techniques to identify that the interaction between LMs and the *E. coli* SC act via an ‘anchor-based’ sequential binding mechanism (Figure 1B–E). Des-amino-Leu-Phe (dLF) was identified as a minimal SC-binding motif that occupies subsite I while maintaining M362 in the “closed” conformation (Figure 1B). In contrast, binding of AcLF stabilizes the "open" conformation of M362, accompanied by formation of a hydrogen bond between the acetylated N-terminal amide NH of the dipeptide and the carbonyl oxygen of G174 (Figure 1C). Binding of AcALDLF to the SC showed electron density for the three C-terminal residues only, which occupied subsite I and the channel while holding M362 in the “open” conformation (Figure 1D). Other pentapeptides, including AcQADLF (Figure 1E), were found to occupy subsite I, the channel and subsite II. We proposed that this series of peptide-bound SC structures captured the order of endogenous LM binding events: i.e., binding of dLF in subsite I mimics the primary binding contact, AcLF demonstrates subsequent formation of the H-bond to G174 with the M362 gate stabilized in the "open" conformation, and AcQADLF demonstrates binding of the N-terminal residues to subsite II by forming a network of hydrogen bonds to the glutamine side-chain amide.

According to the model, subsite I is the first point of contact between LM peptides and the SC, acting as the ‘anchor site’ for inhibitor binding. Two classes of non-peptidic small-molecule inhibitors, a thioxothiazolinine derivative and a biphenyloxime ether.
have previously been shown to occupy subsite I (Supporting Information, Figure S1). Our recent fragment-based discovery efforts identified several new hits that also bind in subsite I.\textsuperscript{22} Herein we report the design, synthesis, biochemical, biophysical and structural characterization of tetrahydrocarbazole (THC)-based inhibitors that mimic the ‘anchoring-based’ sequential binding of LMs to the \textit{E. coli} SC.

![Figure 1](image.png)

**Figure 1.** The sequential binding mechanism of LMs to the SC. (A) The \textit{E. coli} SC structures in complex with pentapeptide AcQADLF (PDB entry 4K3O),\textsuperscript{19} the SC binding-domain of Pol IV (1UNN)\textsuperscript{18} and dsDNA (3BEP).\textsuperscript{17} (B–E) X-ray co-crystal structures showing the binding of dLF, AcLF, AcALDLF (the AcAL-portion is disordered) and
AcQADLF to the SC binding pocket (PDB entries 4K3K, 4K3L, 4K3M and 4K3O, respectively).\textsuperscript{19} Dashed lines in red represent H-bonds/salt bridges. A bridging water molecule is shown as a red sphere. Electrostatic potential surfaces in (A) are shown semi-transparent with blue = positive and red = negative.

**RESULTS**

**A Tetrahydrocarbazole Lead Binds to Subsite I as a Mimic of the Anchor Motif.** In previous work, we showed that the \(R\)-enantiomer of racemic tetrahydrocarbazole (THC) 1 (Figure 2) binds to subsite I of the *E. coli* SC.\textsuperscript{22} Here, we explored optimization of THC binding to subsite I through substitution of the chlorine atom of 1 by other halogens and by shifting the carboxyl group to the 2-position of the cyclohexenyl ring (compounds 1a–j, Figure 2). Our previously reported fluorescence polarization (FP)-based competition assay employing tracer peptide 5-carboxyfluorescein-QLDLF\textsuperscript{20,23,24} was used to quantify binding of 1a–j to the *E. coli* SC.\textsuperscript{19} Inhibition of tracer binding was plotted against inhibitor concentration to generate IC\textsubscript{50} values (Table 1 and Supporting Information, Figure S2), which were subsequently transformed into inhibition constants (\(K_i\)) using the Kenakin correction for ligand depletion (Table 1).\textsuperscript{25} Ligand lipophilicity efficiency (LLE\textsubscript{AT})\textsuperscript{26} values were also calculated to inform inhibitor design, with LLE\textsubscript{AT} values around 0.4 targeted (Table 1).

Racemic 1a was obtained using standard Fisher indole chemistry from commercially available 4-chlorophenyl-hydrazine and (\(\pm\))-3-oxocyclohexanecarboxylic acid (Figure 2). 1a showed a \(K_i\) of 166 \(\mu\)M (Table 1). Esterification of 1a (MeOH, H\textsubscript{2}SO\textsubscript{4} (cat.)) provided
1b, while coupling of 1a with ammonium hydroxide in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxy-succinimide (NHS) afforded amide 1c. LiAlH₄ reduction of 1a gave alcohol 1d. However, none of 1b–d was active (Table 1). The stereochemically pure R and S enantiomers of 1a (i.e., 1e and 1f, respectively) were prepared using enantiomerically pure cyclohexane-3-carboxylic acids. Fluorinated (1g), iodinated (1h) and brominated analogues (1i and 1j) were obtained with the appropriate 4-halophenylhydrazines and chiral cyclohexane-3-carboxylic acids. SC inhibition by 1e–j indicated a preference for the R-enantiomer and chloro/bromo/iodo derivatives (Table 1).

Binding of racemic 1a to the E. coli SC was examined using X-ray crystallography (see crystallographic data in Supporting Information, Table S1). Soaking 1a into an apo-SC crystal resulted in only the R-enantiomer 1e being observed in the electron density of the SC¹e complex (Figure 3), consistent with its higher SC affinity relative to 1f (Table 1). 1e was found to bind in subsite I of one chain (A) of the SC; the asymmetric unit contained a homodimer, chains A and B. Binding of 1e to subsite I of chain B was also observed but a nearby crystal contact led us to consider the observed binding of 1e to chain B to be less relevant (Supporting Information, Figure S3).
Figure 2. THCs (±)-1 and 1a–j. Reagents and conditions: (a) AcOH, reflux, (b) MeOH, H$_2$SO$_4$ (cat.), reflux, (c) (i) EDC/NHS, CH$_2$Cl$_2$, rt., (ii) NH$_4$OH, THF, (d) LiAlH$_4$, THF.

Table 1. Inhibition of the *E. coli* SC by 1a–j

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<th>$K_i$ (µM)</th>
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<td>166</td>
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<td>0.38</td>
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<td>–</td>
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<tr>
<td>1c</td>
<td>&gt;1000</td>
<td>–</td>
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<td>–</td>
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<td>–</td>
</tr>
<tr>
<td>1e</td>
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<td>1f</td>
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<td>0.51</td>
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</tr>
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</tr>
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<tr>
<td>1j</td>
<td>328</td>
<td>182</td>
<td>0.83</td>
<td>0.35</td>
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</table>
Binding of 1e positioned the Cl-substituent deep within a hydrophobic pocket of the SC comprised of V247, V360, L177 and M362; the same region is occupied by the isobutyryl group of dLF (Figure 1B). The cyclohexenyl ring was observed to bind in the same shallow region (containing P242, T172 and L155) occupied by the Phe side chain of dLF. A significant difference between the two complexes was that the M362 gate remained “closed” ($\chi_2 = -173^\circ$, as observed in chain A of the equivalent apo-structure: PDB entry 1MMI$^{29}$) upon binding of dLF (Figure 1B), whereas binding of 1e stabilizes the "open" conformation of the M362 gate ($\chi_2 = -69^\circ$, Figure 3). The "open" conformation of M362 side-chain was previously identified during molecular dynamics simulation of the apo-SC crystal structure, but was only maintained as such when peptide ligands were bound.$^{19, 24}$

Such The carboxylate group of 1e made an H-bond interaction with the phenolic-OH of Y154 and a salt-bridge to the R152 guanidinium group. These additional interactions presumably contributed to the increased SC affinity of 1e ($K_i = 74 \mu M$) relative to dLF ($K_i > 1 \text{ mM}$).$^{19}$ Importantly, the NH group of 1e projected towards the open M362 gate and associated channel, suggesting a synthetically tractable opportunity (i.e., via N-substitution) for directing additional functionality in the direction of subsite II and creating higher potency inhibitors that mimic the endogenous LM binding mechanism.
Figure 3. X-ray co-crystal structure of 1e bound to subsite I of the *E. coli* SC (complex designated SC\textsuperscript{1e}). Carbon atoms of the SC and 1e are shown in orange and non-carbon atoms are colored according to CPK convention. The *apo*-SC structure (PDB entry 1MMI, chain A, shown in yellow) is superimposed onto SC\textsuperscript{1e} for comparison. Pairs of atoms are indicated with dashed red lines. The electron density maps (2mF\text{o}–DF\text{c}) contoured at 1σ are shown in blue wire-basket form.

Inhibitors that Mimic the ‘Anchor-Based’ Sequential Mechanism of LM Binding to the *E. coli* SC. Visual inspection of the SC\textsuperscript{1e} structure (Figure 3) suggested that addition of an acetamide group to the THC nitrogen of 1e would position the appended amide NH in a region where it could potentially make an H-bond to the carbonyl oxygen of G174 – directly analogous to the H-bond observed in LM-SC complexes (Figure 1C–E). Enantiomeric *N*-acetamido-THCs 2a and 2b (carrying Cl-substituents) and the corresponding bromo-enantiomers 4a and 4b were synthesized as a test of this hypothesis. 2a and 2b were obtained in low yields (4–6%) via a 3-step procedure that involved conversion of acids 1e and 1f to ethyl esters (1k and 1l respectively, Figure 4). *N*-alkylation of
the protected THCs with Cs$_2$CO$_3$/ICH$_2$CONH$_2$ and final ester hydrolysis. The low alkylation yields with iodoacetamide led us to pursue an alternative sequence towards the bromo analogs 4a and 4b. Carboxylic acids 1i and 1j were first converted to their respective ethyl esters 1m and 1n and then N-alkylated with tert-butyl bromoacetate in the presence of Cs$_2$CO$_3$ in DMF. Removal of the tert-butyl group using neat TFA followed by EDC/NHS-mediated amidation with aqueous ammonia and final ethyl ester deprotection yielded 4a and 4b in 12% and 17% yields, respectively, over 4 steps.

Compounds 2a and 4a showed higher affinity for the SC than their enantiomeric counterparts 2b and 4b, again reflecting the preference for R-stereochemistry at the ring carboxylic acid (Table 2). The co-crystal structure of the SC with 2a (Figure 5A, complex denoted SC$^{2a}$; see crystallographic data in Supporting Information, Table S2) showed that the binding pose of 2a is similar to 1e (Figure 3). The conformational changes in the side chains of S346 and M362 upon binding of 1e were also observed with 2a and, crucially, it was found that the amide-NH of 2a formed the expected H-bond to the carbonyl oxygen of G174. Additionally, as observed with the similarly sized dipeptide AcLF (Figure 1C), formation of this H-bond coincided with opening of the M362 gate, in agreement with our anchor-based sequential binding model.$^{19}$ The binding mode of 2a suggested that further potency increases might be achieved with N-substituted acetamides, which could similarly trigger opening of the M362 gate and formation of the H-bond to G174 while simultaneously projecting additional functionality across the channel towards subsite II to mimic the SC-binding mechanism of LMs.
Figure 4. Synthesis of N-acetamido-THCs. Reagents and conditions: (a) EtOH, H₂SO₄ (cat.), reflux; (b) (i) ICH₂CONH₂, Cs₂CO₃, DMF, 80°C, (ii) NaOH, EtOH, rt; (c) (i) tert-butyl bromoacetate, Cs₂CO₃, DMF, 80°C, (ii) TFA; (d) (i) EDC/NHS, CH₂Cl₂, rt, (ii) NH₄OHₐq, THF, (iii) LiOH, THF, rt; (e) (i) NH₂-R₂, HATU, DIPEA, DMF, rt, (ii) NaOH or LiOH, EtOH, rt; (f) neat TFA.

Table 2. Inhibition of the E. coli SC by N-Acetamido-THCs

<table>
<thead>
<tr>
<th>IC₅₀ (µM)</th>
<th>Kᵢ (µM)</th>
<th>LogD (pH 7.2</th>
<th>LLEₐ</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>2a</td>
<td>368</td>
<td>204</td>
<td>−0.75</td>
<td>0.40</td>
</tr>
<tr>
<td>2b</td>
<td>888</td>
<td>493</td>
<td>−0.75</td>
<td>0.38</td>
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<td>4a</td>
<td>246</td>
<td>137</td>
<td>−0.73</td>
<td>0.41</td>
</tr>
<tr>
<td>4b</td>
<td>285</td>
<td>158</td>
<td>−0.73</td>
<td>0.41</td>
</tr>
<tr>
<td>5a</td>
<td>85</td>
<td>47</td>
<td>−4.76</td>
<td>0.61</td>
</tr>
</tbody>
</table>
Aspartate or glutamate residues are often present in SC-binding LMs, serving as bridging residues that occupy the channel region between subsites I and II (Figure 1D–E).\textsuperscript{15, 19, 24} We predicted that glycinamides 5a and 5b (Figure 4) would position their Gly carboxylate groups into the region occupied by LM Asp and Glu side chains. To test this, 5a and 5b were prepared in modest yields (10–40%) from their precursor ethyl esters 3a and 3c by first coupling glycine methyl ester using standard amide coupling conditions (HATU, DIPEA, DMF) and then simultaneously hydrolyzing the methyl and ethyl esters with base. The precursor ester 3c was prepared as for 3a from 4-chlorophenylhydrazine in
59% yield over 4 steps (Figure 4). 5a and 5b both contained R-configured carboxylates at the 2-position. The S-enantiomers of 5a and 5b (and other inhibitors) were not investigated beyond this point due to the lower SC affinity observed (Tables 1 and 2). The bromo-acid analog 5b showed higher potency ($K_i = 11 \text{ } \mu\text{M}$, Table 2) relative to the corresponding primary amide 4a ($K_i = 137 \text{ } \mu\text{M}$) and the chloro-analog 5a ($K_i = 47 \text{ } \mu\text{M}$). These results led us to focus subsequent efforts on bromo-THCs.
Figure 5. X-ray co-crystal structure of (A) 2a, (B) 5b and (C) 5l bound to the *E. coli* SC (complexes designated SC\textsuperscript{2a}, SC\textsuperscript{5b} and SC\textsuperscript{5l}, respectively). The complexes are represented with carbon atoms shown in orange and non-carbon atoms following CPK convention. The *apo*-SC structure (PDB entry 1MMI, chain A, shown in lemon) is superimposed on each complex for comparison. Inhibitor electron density maps (2mF\textsubscript{o}–DF\textsubscript{c}) contoured at 1σ are shown in blue wire-basket form.
A co-crystal structure of the SC with 5b (Figure 5B, complex denoted SC$^{5b}$; see crystallographic data in Supporting Information, Table S1) showed that 5b made all of the same SC interactions as 2a (Figure 5A). The appended glycine carboxylate of 5b, however, made additional hydrogen bond/salt bridge contacts with the side chains of R152 and H175, thus explaining its higher potency relative to its parent amide 4a. As expected, the glycyl group occupied the channel region between subsites I and II, mimicking the channel-binding Asp residues in LMs (Figure 1D, E). 5c–i (Figure 4), synthesized in variable yields (4–64%) using similar methods, were found to have reduced potency relative to 5b (Table 2). Amino acid-containing compounds 5j–m were examined next to explore whether side chains appended to the 5b glycine α-carbon could occupy subsite II. 5l and 5m were prepared in moderate yields (30–57%) via amide coupling reactions of D- and L-phenylalanine methyl esters with 3a, respectively, followed by global methyl/ethyl ester deprotection using base (Figure 4). Both 5l and 5m ($K_i = 17$ and $15 \mu M$, respectively, Table 2) showed similar SC affinities to 5b ($K_i = 11 \mu M$, Table 2).

A co-crystal structure of the SC with 5l (Figure 5C, complex denoted SC$^{5l}$; crystallographic data in Supporting Information, Table S1) showed that the overall binding pose of 5l was similar to that of 5b (Figure 5B). 5l extended to the edge of subsite II where its phenyl ring occupied part of the space filled by LM peptide sub-motifs (Figure 1E). The M362 side chain adopted alternate conformations upon binding of 5l, one where it displayed the usual “open” state whilst in the other it was shifted towards subsite II due to an interaction with the phenyl ring.
The binding of inhibitors 1e, 2a, 5b and 5l to the *E. coli* SC closely matched our ‘anchor’-based LM sequential binding mechanism. Compound 1e is positioned as an ‘anchor’ in subsite I. Having the same THC moiety anchored in subsite I, compounds 2a, 5b and 5l maintained the M362 gate in the open conformation and made the H-bond interaction with G174 – features uniformly observed in LM-bound SC structures. It therefore seems likely that binding of 2a, 5b and 5l (and probably other THC-based inhibitors) would invoke the sequential binding mechanisms of LMs; i.e., where the THC group makes initial contact with subsite I, captures the "open" state of the M362 gate and then redirection of the appended groups occurs leading to formation of the G174 H-bond. This assertion is supported by the poses adopted by 2a and 5l when bound to the SC chain B (Supporting Information, Figure S3). Inhibitor binding to chain B beyond subsite I is impeded by the presence of the SC symmetry partner – a crystallographic artefact. In these structures, 2a and 5l positioned their THC moieties in subsite I but their appended groups were shunted away from the channel and subsite II into solvent by the crystallographic symmetry partner.

**Ligand-Induced Loop Rearrangement Creates an Additional Binding Pocket.** The observation of similar SC binding affinities for diastereomers 5l and 5m (containing D- or L-Phe moieties, respectively) led us to investigate the binding pose adopted by 5m. SC/5m co-crystal structures were solved (denoted SC\textsuperscript{5m}, Figure 6A; crystallographic data in Supporting Information, Table S2) and the apo-SC structure with similar cell dimensions was also solved for comparison (denoted SC\textsuperscript{apo}; crystallographic data in Supporting
Information, Table S2). $5m$ was observed to bind to chain B of this SC crystal with its THC unit occupying subsite I, as observed with other THCs. The symmetry partners that had occupied the channel region and subsite II of chain B in other SC crystals of smaller cell dimensions were not observed with this structure. The binding of $5m$ caused similar conformation changes in the side chains of S346 and M362 to those observed in other THC-SC structures; however, a dramatic rearrangement in the loop region comprising H148–Y154 was induced upon binding of $5m$ (Figure 6A; additional electron density maps highlighting the differences between the loop regions of SC$^{apo}$ and SC$^{5m}$ are provided in Supporting Information, Figure S4). While the H148 and Y154 residues at the start and end of the loop had undergone minor changes in their side chain conformations, the residue pair of Q149 and D150 had exchanged positions. The residue pair of R152 and Y153 had also exchanged positions and V151, located in the middle of the loop, had moved to accommodate these changes.
Figure 6. Co-crystal structure of 5m bound to the E. coli SC. (A) SC\textsuperscript{5m} is displayed with carbon atoms in orange and non-carbon atoms colored according to CPK convention. The SC\textsuperscript{apo} structure is superimposed onto SC\textsuperscript{5m} for comparison (carbon atoms colored yellow and non-carbon atoms in CPK colors). Hydrogen bonds are indicated with dashed red lines. Purple and green arrows indicate movement of SC residues in the SC\textsuperscript{apo} vs. SC\textsuperscript{5m} structure. Electron density maps (2mF\textsubscript{o}–DF\textsubscript{c}) contoured at 1σ are shown in blue wire-basket form. (B and C) Electrostatic potential surfaces of the binding sites in SC\textsuperscript{apo} and SC\textsuperscript{5m} shown in semi-transparent form (blue = positive; red = negative). (D and (E) Struc-
tural overlap of SC<sup>5m</sup> and dsDNA-bound SC (PDB entry 3BEP) with carbon atoms in orange and cyan respectively. Atoms of DNA molecules are in CPK colors and the nucleic acid bases are also represented as blocks (A: red, G: green, T: yellow, C: blue). The dsDNA axis is directed at the viewer in (D) and the system is rotated 90° clockwise in (E).

The loop rearrangement significantly altered the surface geometry and electrostatics in this region of the SC (Figures 6B and C). The charged area originally occupied by the side chains of R152 and D150 and the surrounding residues G174, Y154 and H175 in SC<sub>apo</sub> were replaced by neutral residues Y153 and Q149 in SC<sup>5m</sup> to accommodate the hydrophobic phenyl ring of 5m. We denote the new ligand-induced binding area, comprising G174, Y154, Y153, Q149 and H175 as “subsite III”. It is of note that the phenyl group of 5m occupied only half of subsite III, leaving the area between H175 and Q149 vacant and potentially available for further targeting.

Sequence alignments comparing the LM binding pockets and H148–Y154 loop regions of SCs from representative Gram-negative (E. coli, Acinetobacter baylyi, A. baumannii and Pseudomonas aeruginosa) and Gram-positive (Staphylococcus aureus, Bacillus subtilis, Streptococcus pneumoniae and S. pyogenes) species (Supporting Information, Figure S5) show that the switching residue pairs in the loop region (i.e., Q149–D150 and R152–Y153), along with V151, are well conserved across the Gram-negatives, but less so among the Gram-positives. This suggests that the ligand-induced loop rearrangement may be a feature common to Gram-negative species. The conformational change is also of interest because the rearranged loop is located at the inner edge of the SC dimer in the
region through which dsDNA passes (Figure 4D, E). The ~10 Å movement of V151 towards the center of the ring positioned it where dsDNA would be expected to bind (see PDB entry 3BEP). The movements in several residues involved in both the binding of 5m and ssDNA/dsDNA shows that this region is open to remodeling by ligands. In addition to inhibiting LM binding, 5m may also act as an inhibitor of the SC/DNA interaction. This hypothesis may in part be corroborated by the *E. coli* SC-DNA complex crystal structure, which demonstrated side-chain movement of Q149 upon dsDNA binding due to steric hindrance, and residues Y153 and Y154 interacting with a nearby ssDNA segment from a symmetry-related molecule.

**Dissociation Constants of Complexes of THC Derivatives with the *E. coli* SC.**

Thermodynamic data for the binding of THCs 5a, 5b, 5l and 5m to the *E. coli* SC were obtained using isothermal titration calorimetry (ITC). In all cases the observed dissociation constants, \( K_d \) (Table 3 and Supporting Information Figure S6) matched well the \( K_i \) values obtained from the fluorescence polarization assays (Table 2; Supplementary Information, Figure S2). The thermodynamic profiles (\( \Delta H \) and \(-T\Delta S\) values) for each inhibitor were also similar, consistent with their closely related binding poses in subsite I. Interestingly, the loop rearrangement induced in the SC upon binding of 5m did not significantly alter the enthalpy or entropy of binding relative to other inhibitors.
Table 3. ITC Data (298 K) for the Binding of THC Derivatives to the E. coli SC

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<th>$\Delta G$ (kcal/mol)</th>
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<tr>
<td>5a</td>
<td>-13.2 ± 0.16</td>
<td>6.8</td>
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<td>24 ± 2</td>
</tr>
<tr>
<td>5b</td>
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<td>8.5</td>
<td>-6.6</td>
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<tr>
<td>5l</td>
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<td>5.3</td>
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<td>13 ± 1</td>
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<tr>
<td>5m</td>
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<td>6.8</td>
<td>-6.9</td>
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</table>

THC Derivatives Inhibit in vitro DNA Replication. DNA replication assays were used to demonstrate inhibition of E. coli SC function by THC derivatives 1e, 1i, 2a, 4a, 5a, 5b, 5l and 5m. Briefly, circular ssDNA with an RNA primer was used as template in a minimized reaction system containing the SC, single-stranded DNA-binding protein (SSB), the Pol III α subunit and the reconstituted $\gamma_3\delta\delta'$ clamp loader complex. In this system purposely minimized to avoid potentially confounding effects of inhibitors on other protein–protein interactions in more efficient full-replisome systems, the SC is loaded onto the primed DNA by the clamp loader complex, which is subsequently released. Binding of the α subunit leads to extension of the primer through incorporation of dNTPs using the primed ssDNA as template (see Supporting Information, Figures S7 and S8 for a schematic representation of the assay and a control experiment). Binding of the DNA Pol III δ and α subunits to the SC is essential for DNA replication to occur in this system and is impaired by the presence of inhibitors.
hibition of SC binding leaves residual ssDNA templates and incomplete dsDNA products, whereas successful synthesis yields complete dsDNA products (Figure 7).

![DNA replication image](image)

**Figure 7.** Inhibition of *E. coli* SC-mediated *in vitro* DNA replication by **1e, 1i, 2a, 4a, 5a, 5b, 5l and 5m**. DNA ladders indicate 8, 6, 5, 4 and 3 kilobase pairs of DNA in each panel (top to bottom). Ladder = DNA molecular size standards. Circles with a short line above represent RNA-primed ssDNA templates. Two concentric circles represent completed dsDNA synthesis products.

Compounds **1e, 1i, 2a** and **4a** inhibited DNA replication at concentrations >125 µM (Figure 7), consistent with their relatively weak SC affinities (*K*_i* = 74–204 µM, Tables 1 and 2). Compounds **5a** (*K*_i* = 47 µM) inhibited DNA replication at concentrations >63 µM, while **5b** (*K*_i* = 11 µM), **5l** (*K*_i* = 17 µM) and **5m** (*K*_i* = 15 µM) all showed inhibitory effects at 31 µM, consistent with their higher SC affinities.

**DISCUSSION**
A characteristic feature of linear motif (LM) recognition by the *E. coli* sliding clamp (SC) is separation of the LM binding pocket on the SC surface into two distinct subsites (subsites I and II), with an intervening channel region gated by the side chain of M362. Subsite I acts as the anchor site for binding of ‘anchor motifs’ in the consensus sequences of partner proteins. All SC inhibitors identified to date bind in subsite I. Our sequential binding model describes how binding events at the channel region and subsite II occur only after ligands are first ‘anchored’ into subsite I. Tetrahydrocarbazole (THC) derivatives 1e and 1i were identified as ligands that act as anchor moieties in subsite I, while at the same time positioning their THC nitrogen in an orientation that allowed additional functionality to be added and projected into the channel towards subsite II, thus matching the binding mechanism of LM consensus peptide ligands. The hydrogen bond with G174 and the “open” conformation of the M362 gating residue, which are both consistently observed upon LM peptide binding, were reproduced by members of the *N*-linked THCs, exemplified by 2a, 5b and 5l (Figure 5). Compound 5l was found to occupy subsite I, the channel region and the edge of subsite II, providing valuable insights for future work to, for example, extend inhibitors farther into subsite II by substituting the phenyl ring of the D-phenylalanine moiety or by replacing it with other functionalized D-amino acids.

The binding of 5m to the *E. coli* SC induced a loop rearrangement that resulted in positional switching of residue pairs Q149–D150 and R152–Y153 and a 10 Å movement of V151. To our knowledge, no peptides or synthetic entities that bind the SC (including the clamp-loader complex, which can pry open the SC dimer ring) have been observed to
exert such allosteric effect. This ligand-induced rearrangement revealed a hitherto unreported binding pocket, which we denote ‘subsite III’. Compound 5m did not form the hydrogen bond with G174 observed with other THC derivatives (Figure 6A), nor did its carboxylate group make interactions with R152 and H175 (as observed with 5b and 5l; Figure 5B and C). Nonetheless, 5m displayed similar SC affinity to 5b and 5l, suggesting that the SC conformational changes and occupancy of subsite III by 5m contributed favorably to binding (Figure 6C). Subsite III therefore appears as an attractive ‘ligand-induced’ site for targeting through further substitution of 5m or related structures. Compounds of this type have potential to inhibit the SCs of Gram-negative bacteria in particular, due to their well conserved subsite I and loop residues H149–Y153. The possibility that 5m and associated derivatives might also modulate SC–DNA interactions is intriguing but remains to be investigated.

THC derivatives 5b, 5l and 5m showed >10-fold increases in SC affinity relative to initial hit 1a, and also have increased LLE_AT values. The previously described thioxothiazolinine derivative and biphenyloxime derivative SC inhibitors (Supporting Information, Figure S1) bound to subsite I and showed affinities >10 µM. THC derivatives 5l and 5m displayed similar SC affinities achieved through expanded binding site coverage to compensate reduced ligand efficiency, with 5l extending to the edge of subsite II and 5m revealing the new subsite III. Both compounds provide synthetically accessible possibilities for evolving further generations of inhibitors that fully occupy subsites I and II or III and show increased potency.
Binding of these \( N \)-substituted THCs was in good agreement with the anchor-based sequential binding mechanism proposed for SC-binding LM peptides. The substituted THC anchor motifs occupied subsite I first, followed by positioning of the rest of the ligand. This observation may be generally applicable for development of inhibitors of LM binding, since other LMs may also possess key ‘anchor’ residues that drive binding to partner proteins. Similar inhibitor design strategies may be successful when applied in evolutionarily related and/or biochemically similar systems. For example, the eukaryotic SC homolog, PCNA, that mediates interactions in eukaryotic DNA replication via recognition of a Qxx[I/L]xxF motif, has been suggested as an anticancer target. Targeting ‘anchor sites’ and adjacent areas in this (and other) systems, after delineating the mechanisms of LM recognition, may serve as an effective strategy for inhibitor design.

**MATERIALS AND METHODS**

**Chemo-informatics, Bioinformatics and Organic Synthesis.** Compounds were \( \geq \) 95% pure as confirmed by \(^1\)H NMR. Full details are given in the Supporting Information.

**Protein Expression and Purification.** Expression and purification of the *E. coli* SC, Pol III \( \alpha \) subunit, SSB and the \( \gamma_3\delta\delta' \) clamp loader complex were as described previously.

**Crystallization and X-Ray Data Collection.** Crystals of the *E. coli* SC were grown at 285 K by the hanging-drop vapor diffusion method. The hanging drop was composed of 1 µl of sliding clamp (53 mg/mL in 10 mM Tris-HCl pH 7.2, 1 mM dithiothreitol, 1 mM EDTA and 15% glycerol) mixed with an equal volume of reservoir solution consisting of
100 mM MES pH 6.5, 100–150 mM CaCl$_2$ and 25–30% (v/v) PEG400. The reservoir volume was 1 mL. SC crystals were transferred to a CaCl$_2$-free reservoir and ligands were soaked into the crystal at 2–5 mM in reservoir solution with <10% DMSO. All crystals were mounted using MiTeGen loops on pins with magnetic caps. For in-house data collection, crystals were flash-frozen at 100 K using an Oxford Cryostream. Diffraction data were collected using a Mar345 desktop beamline using CuK$_\alpha$ X-rays from a Rigaku 007HF rotating anode generator with Varimax optics. For synchrotron data collection, the SSRL automated mounting system (SAM) was used. Mounted crystals were flash-frozen in liquid nitrogen and placed in the SAM cassettes. Diffraction data were collected at 100 K at the Australian Synchrotron, Beamline MX1 using X-rays of wavelength 0.95 Å.

**Data Processing, Structure Solution and Refinement.** Crystal data sets were integrated, merged and scaled with HKL2000$^{39}$ or MOSFLM and SCALA.$^{40}$ The structures were solved by molecular replacement with CCP4 using the Protein Data Bank entry 1MMI$^{29}$ or 4K3S$^{19}$ as the starting model. Iterative cycles of model building and refinement were performed in COOT$^{41}$ and REFMAC5. $^{42}$

**Fluorescence Polarization Assay.** Experiments and data processing protocols were reported previously.$^{19}$ All FP experiments were conducted using a POLARstar Omega plate reader with non-treated black sterile 96-well plates (Greiner, USA). The buffer contained 10 mM HEPES pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 0.07% Nonidet P-40 and 5% DMSO. The fluorescent tracer used was N-fluorescein (FAM)-QLDLF-OH (GL Biochem, China), which has a $K_d$ of 70 nM for SC monomers. For the competition assay, 10
nM peptide and 50 nM sliding clamp (as monomers) were used. Blank control (buffer), negative control (buffer and the peptide) and positive control (buffer, peptide and the sliding clamp) were used for data normalization. Data are shown as % inhibition relative to maximum, calculated as the decrease in polarization from maximum divided by the background-subtracted total polarization. Experiments were carried out in duplicate. Curves were fit using GraphPad Prism v5.01 (GraphPad Software, USA) and data are presented as "best-fit" values. Dose-response curve fitting was applied to competition assays with variable slope.

**Isothermal Titration Calorimetry.** ITC used a MicroCal Auto-iTC200 calorimeter (GE Healthcare) at 298 K. The SC was dialyzed against a buffer (10 mM HEPES pH 7.2, 0.15 M NaCl and 1 mM EDTA) and the same buffer was used to dissolve the compounds. The feedback mode was “high” with a reference power setting of 10 µcal/s. The cell was stirred at 1000 rpm and the thermostat set at 25 °C. Experiments were conducted with 19 injections of 2 µL over 4s with 200s intervals between injections. The first injection (of 0.4 µL) was discarded in all cases. Compounds (0.7-1 mM) were titrated in sequential injections into 75 µM SC monomer. Data were corrected for control experiments with compounds titrated into buffer only. Data analysis was carried out with Origin 7.0 using one-site binding data fitting.

**DNA replication assay.** The RNA-primed DNA template was prepared in advance by mixing 35 nM wild-type M13 ssDNA\textsuperscript{26} with 1 µM oligoribonucleotide (5’-UAUGUACC-CGGUUGAUAUCAAGAAAAAGCCCA; GeneWorks, Australia) in 30
mM Tris-HCl pH 7.6, 15 mM MgCl$_2$, 130 mM NaCl and 0.1 mM EDTA for 10 min at 55 °C and cooling to room temperature over a period of 8 h. The DNA replication assays contained: 20 mM Tris-HCl pH 7.6, 10 mM MgCl$_2$, 0.8 mM ATP, 8.4 mM dithiothreitol, 0.6 mM of each dNTP, 50 nM Pol III α subunit, 700 nM SSB (as tetramers), 210 nM Pol III β sliding clamp (as dimers), 42 nM γ$_3$δδ’ clamp loader complex, 120 mM NaCl and 3 nM RNA primed DNA template, in a volume of 15 µL. Inhibitors (peptides or THC derivatives) were dissolved in DMSO and diluted in series 2-fold (in 50% v/v DMSO) before being added (0.5 µL) to the assay mixture at 0°C. The final DMSO concentration was 3.4% (v/v) in all assays. The assay mixtures were treated at 30 °C for 60 min before being quenched by the addition of EDTA to 150 mM and SDS to 1% (w/v). The DNA products were separated by 0.7% agarose gel electrophoresis in TAE buffer (80 mM Tris, 40 mM acetic acid, and 4 mM EDTA) and then stained with 10,000-fold diluted SYBR Gold (Life Technologies) for 60 min. The DNA products were visualized using a UV transilluminator.

ASSOCIATED CONTENT

Supporting Information

Supplementary figures, crystallographic data, supplementary methods of chemoinformatics, bioinformatics and organic synthesis. This material is available free of charge via the Internet at http://pubs.acs.org.

Accession codes
The atomic coordinates and structure factors for SC<sup>1e</sup>, SC<sup>2a</sup>, SC<sup>5b</sup>, SC<sup>5l</sup>, SC<sup>apo</sup> and SC<sup>5m</sup> have been deposited in the Protein Data Bank with accession codes 4OVF, 4OVG, 4OVH, 4PNU, 4PNV and 4PNW, respectively.

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**Author Contributions**

#Z.Y. and L.R.W. contributed equally to this work.

**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

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**ABBREVIATIONS USED**
dNTP, deoxyribonucleoside triphosphate; dsDNA, double-stranded DNA; dLF, desamino-Leu-Phe; DMF, dimethylformamide; DIPEA, N,N-diisopropylethylamine; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; FP, fluorescence polarization; HATU, N-[(dimethylamino)-1H-1,2,3-triazolo-[4,5-b]pyridin-1-ylmethylene]-N-methylmethanaminium hexafluorophosphate N-oxide; IC$_{50}$, half-maximum inhibitory concentration; $K_i$, inhibition constant; LogD, distribution coefficient; LE, ligand efficiency; LLE$_{AT}$, ligand lipophilicity efficiency; LM, linear motif; NHS, N-hydroxysuccinimide; PCNA, proliferating cell nuclear antigen; PDB, protein data bank; Pol, polymerase; SC, sliding clamp; ssDNA, single-stranded DNA; TFA, trifluoroacetic acid; THC, tetrahydrocarbazole.

REFERENCES


(23) Dohrmann, P. R.; McHenry, C. S. A bipartite polymerase-processivity factor interaction: only the internal β binding site of the α subunit is required for processive replication by the DNA polymerase III holoenzyme. *J. Mol. Biol.* **2005**, *350*, 228–239.


Bacterial Sliding Clamp Inhibitors that Mimic the Sequential Binding Mechanism of Endogenous Linear Motifs

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‡School of Environmental and Life Sciences, The University of Newcastle, Callaghan, New South Wales 2308, Australia

Supporting Information

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Supplementary Results

**Figure S1.** X-ray co-crystal structures of the *E. coli* SC in complex with (A) a thioxothiazolinine derivative (PDB entry 3D1G)\(^1\) and (B) a biphenyloxime ether derivative (PDB entry 3QSB).\(^2\) Carbon atoms are colored orange and non-carbon atoms follow CPK convention. Both inhibitors occupy subsite I.
Figure S2. Dose-response curves of representative THC analogs inhibiting the *E. coli* SC, as measured by fluorescence polarization.
Table S1. Data collection and refinement statistics for X-ray co-crystal structures of the *E. coli* SC in complex with compounds 1e, 2a, 5b and 5l

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<th>Name</th>
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<th>SC&lt;sup&gt;3d&lt;/sup&gt;</th>
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<td>4OVF</td>
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<td>4O VH</td>
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**Data collection**

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<td>Completeness (%)</td>
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<tr>
<td>Multiplicity</td>
<td>3.6 (3.5)</td>
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**Refinement**

| Resolution (Å) | 34.61–2.05 (2.10–2.05) |
| R<sub>work</sub> / R<sub>free</sub> (%) | 21.0 (19.7) / 27.0 (29.9) |
| RMS deviations |
| Bond lengths (Å) | 0.0056 |
| Bond angles (°) | 1.0960 |
| B-factors |
| main chain | 22.6 |
| sidechain & water | 26.4 |
| ligand* | 32.7 |
| Ramachandran Plot Outliers | 0.43% |

Values for data in the highest resolution shell are given in parentheses.

Diffraction data were collected using a Rigaku 007HF X-ray generator producing Cu Kα X-rays (wavelength of 1.5418 Å) and Mar345dtb area detector. Diffraction data were processed using HKL2000.<sup>3</sup>

*Ligand refers to the compounds bound to SC chain A.
Figure S3. X-ray co-crystal structures of the *E. coli* SC showing chain B in complex with compounds 1e, 2a and 5l. Carbon atoms of SC chain A/B and the bound compounds 1e, 2a and 5l are colored orange/magenta respectively. All other atoms are colored according to the CPK convention. The SC symmetry partner is shown in gray. SC chain A is superimposed with chain B in the enlarged views and the electrostatic potential surfaces of chain B are shown with blue = positive and red = negative. Electron density maps (2mFo–DFc) contoured at 1σ are shown in blue wire-basket form.
Table S2. Data collection and refinement statistics for the X-ray crystal structures of apo-E. coli SC (SC\textsuperscript{apo}) and E. coli SC in complex with compound 5m (SC\textsuperscript{Sm}), respectively

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Data collection

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<td>Multiplicity (%)</td>
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Refinement

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R.M.S. deviations

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B-factors

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Ramachandran Plot Outliers

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<td>0.29%</td>
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Values for data in the highest resolution shell are given in parentheses.

Diffraction data were collected using a Rigaku 007HF X-ray generator producing Cu Kα X-rays (wavelength of 1.5418 Å) and Mar345dtb area detector. Diffraction data were processed with HKL2000.\textsuperscript{3}

*Ligand refers to compounds bound to SC Chain B.
Figure S4. Stereo view of the loop rearrangement induced upon binding of compound 5m to the *E. coli* SC. (A) The H148–Y154 loop region of SC<sup>apo</sup>. Carbon atoms are shown in yellow. All other atoms are colored according to CPK convention. (B) The H148–Y154 loop region of SC<sup>5m</sup>. Carbon atoms are colored orange. All other atoms are colored according to CPK convention. Electron density maps (2mF<sub>o</sub>−DF<sub>c</sub>) contoured at 1σ are shown in blue wire-basket form.
Figure S5. Sequence Alignment of SCs from four representative Gram-negative (Escherichia coli, Acinetobacter baylyi, Acinetobacter baumannii and Pseudomonas aeruginosa) and four Gram-positive bacterial species (Staphylococcus aureus, Bacillus subtilis, Streptococcus pneumoniae and Streptococcus pyogenes). Red box indicates the H148–Y154 loop region. The switching pairs of residues, i.e., Q149–D150 and R152–Y153, are highlighted in purple and V151 in green for the Gram-negative species. Residues comprising subsites I and II are highlighted in yellow. Numbering is based on the E. coli SC sequence.
Figure S6. Isothermal titration calorimetry data for the binding of (A) 5a, (B) 5b, (C) 5l and (D) 5m to the *E. coli* SC.
**Figure S7.** Schematic representation of the *in vitro* DNA replication assay.

**Figure S8.** Control *in vitro* DNA replication assay. Molecular sizes (in bp) corresponding to bands in the DNA ladder are shown. The circle with dashed line above represents primed ssDNA template. Two concentric circles represent completed dsDNA replication products.
**Supplementary Methods**

**Chemo-informatics.** LogD values at pH 7.2 were calculated using Accord for Excel 6.2 (Accelrys). Calculations of Ligand Lipophilicity Efficiency (LLE$_{AT}$) followed the published methods$^4$ and are summarized below:

\[ \Delta G^* = \Delta G - \Delta G_{lip} \]

\[ = RT\ln(K_i) + RT\log D \]

LLE$_{AT} = 0.11 - \Delta G^*/HAC$

\[ \Delta G: \text{ difference in Gibbs free energy; } K_i: \text{ inhibition constant; } HAC: \text{ heavy atom count; LogD: distribution coefficient at pH 7.2.} \]

**Bioinformatics.** Sequence alignments of bacterial sliding clamps (NCBI IDs: YP_859300.1, WP_004930066.1, ZP_08441263.1, NP_064722.1, NP_373240.1, WP_003242509.1, YP_815419.1 and AAF98349.2) were carried out with COBALT.$^5$

**Chemistry – General.** $^1$H and $^{13}$C NMR spectra were acquired on a Varian Mercury 300 MHz, Varian Inova 500 MHz or VNMRS 500 MHz spectrometer. Chemical shifts (δ) are reported in ppm relative to the solvent and coupling constants (J) are in Hz. Electrospray ionisation (EI) low resolution mass spectra (LRMS) were recorded on a Waters Micromass Platform LCZ spectrometer. High resolution mass spectra (HRMS) were recorded on a Waters Xevo spectrometer. Chemical shifts (δ) are in ppm relative to the solvent. Electrospray ionisation (ESI) high resolution mass spectra were obtained on a Waters Micromass Platform LCZ spectrometer. High resolution mass spectra (HRMS) were recorded on a Waters Xevo spectrometer. Optical rotations were measured on a Jasco P-2000 polarimeter. TLC analysis was performed using pre-coated Merck silica gel 60 PF$_{254}$ aluminium sheets. Flash column chromatography was performed using Davisil silica gel (40–63 μm). Petrol refers to petroleum spirits of bp 40–60°C. All compounds examined showed ≥ 95% purity by $^1$H NMR and HPLC-MS.

(±)-6-Chloro-2,3,4,9-tetrahydrocarbazole-2-carboxylic acid (1a).$^6,7$ To a solution of 4-chlorophenylhydrazine hydrochloride (305 mg, 1.70 mmol) in glacial acetic acid (2 mL) was added 3-oxo-cyclohexanecarboxylic acid (237 mg, 1.67 mmol) in glacial acetic acid (2 mL) and the mixture heated at reflux overnight. The reaction mixture was cooled and the resulting precipitate collected by vacuum filtration and washed with cold water. The resultant solid was purified by silica gel column chromatography (25:75:0.5 to 50:50:0.5 Et$_2$O/petrol/AcOH) followed by recrystallization from EtOH/H$_2$O to give 1a (42 mg, 10% yield) as a yellow powder: mp 242–244°C (lit.$^6$ 249–250°C); $^1$H NMR (DMSO-d$_6$, 500 MHz): δ 1.80–183 (1H, m), 2.14–2.16 (1H, m), 2.56–2.92 (5H, m), 6.97 (1H, d, J = 8.0 Hz), 7.24 (1H, d, J = 8.5 Hz), 7.34 (1H, s), 10.90 (1H, s), 12.32 (1H, s); $^{13}$C NMR (DMSO-d$_6$, 125 MHz): δ 19.4, 25.1, 25.7, 39.3, 107.6, 112.0, 116.5, 119.9, 122.8, 128.1, 134.3, 135.2, 175.97; LRMS (ES$^+$) m/z: 272.2 [M+Na]$^+$; HRMS (ASAP$^+$) calcd. for C$_{13}$H$_{13}$NO$_2$Cl [M+H]$^+$ 250.0635, found 250.0628.

(±)-Methyl 6-chloro-2,3,4,9-tetrahydro-1H-carbazole-2-carboxylate (1b).$^6$ To a solution of 1a (50 mg, 0.20 mmol) in MeOH (1.5 mL) was added concentrated H$_2$SO$_4$ (200 μL) and the mixture heated at reflux overnight. The resulting solution was cooled, concentrated to dryness, treated with saturated aqueous NaHCO$_3$ and extracted with EtOAc (3 x 10 mL). The combined organic extracts were
washed with water (10 mL) and brine (10 mL), dried over anhydrous MgSO₄ and concentrated. The crude residue was recrystallized from MeOH/H₂O to give 1b (16 mg, 31% yield) as a beige powder: mp 164–166 °C (lit. 175–176 °C); ¹H NMR (DMSO-d₆, 500 MHz): δ 1.81–1.86 (1H, m), 2.15–2.17 (1H, m), 2.59–2.71 (2H, m), 2.89–2.96 (3H, m), 3.65 (3H, s), 6.98 (1H, dd, J = 18.5, 2.0 Hz), 7.26 (1H, d, J = 9.0 Hz), 7.35 (1H, d, J = 1.0 Hz), 10.94 (1H, brs); ¹³C NMR (DMSO-d₆, 125 MHz): δ 19.4, 25.1, 25.7, 39.1, 51.6, 107.6, 112.0, 116.6, 120.0, 122.8, 128.0, 134.3, 134.8, 174.7; LRMS (ES⁺) m/z: 264.1 [M+H⁺]; HRMS (ES⁺) calcd. for C₁₄H₁₅NO₂Cl [M+H⁺]⁺: 264.0791, found 264.0791.

(±)-6-Chloro-2,3,4,9-tetrahydro-1H-carbazole-2-carboxamide (1c). ⁷ To a solution of 1a (75 mg, 0.30 mmol) in dry CH₂Cl₂ (2 mL) containing a few drops of DMF was added N-hydroxysuccinimide (59 mg, 0.51 mmol) and N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (97 mg, 0.51 mmol) and the mixture stirred for 2.5 h at room temperature. The resulting suspension was diluted with water and extracted with CH₂Cl₂ (3 x 10 mL). The combined organic extracts were dried over anhydrous MgSO₄ and concentrated. The resultant residue was redissolved in THF (1 mL), NH₄OH (28%; 1 mL) added and the mixture stirred for 2 h. The resulting orange solution was diluted with water and extracted with EtOAc (3 x 20 mL). The combined organic extracts were washed with brine (10 mL), dried over anhydrous MgSO₄ and concentrated. Trituration of the residue with CH₂Cl₂ gave 1c (23 mg, 31% yield) as an off-white powder: mp 194–196°C (lit. 169–175 °C); ¹H NMR (DMSO-d₆, 500 MHz): δ 1.69–1.75 (1H, m), 2.05–2.09 (1H, m), 2.53–2.64 (2H, m), 2.71–2.87 (3H, m), 6.87 (1H, br s), 6.97 (1H, dd, J = 8.0, 1.3 Hz), 7.24 (1H, d, J = 8.5 Hz), 7.35 (1H, s), 7.42 (1H, br s), 10.90 (1H, s); ¹³C NMR (DMSO-d₆, 125 MHz): δ 19.9, 25.5, 26.7, 40.4, 107.6, 112.0, 116.5, 119.9, 122.8, 128.1, 134.3, 135.7, 176.5; LRMS (ES⁺) m/z: 271.11 [M+Na⁺]; HRMS (ES⁺) calcd. for C₁₃H₁₃N₂OCINa [M+Na⁺]: 271.0614, found 271.0616.

(±)-(6-Chloro-2,3,4,9-tetrahydro-1H-carbazol-2-yl)methanol (1d). ⁶ To a solution of 1a (75 mg, 0.30 mmol) in dry THF (2 mL) was added portion-wise LiAlH₄ (35 mg, 0.93 mmol) and the mixture stirred at room temperature for 3 h. A saturated solution of sodium potassium tartrate in water was then added and the mixture stirred for 30 min before being extracted with EtOAc (3 x 20 mL). The combined organic extracts were washed with brine (10 mL), dried over anhydrous MgSO₄ and concentrated. The resulting residue was triturated with CH₂Cl₂ to give 1d (30 mg, 20% yield) as a yellow powder: mp 152–154°C (lit. 168–169 °C); ¹H NMR (CD₃OD, 500 MHz): δ 1.50–1.52 (1H, m), 2.04–2.05 (2H, m), 2.43–2.48 (1H, m), 2.59–2.61 (1H, m), 2.71–2.74 (1H, m), 2.81–2.85 (1H, m), 3.55–3.62 (2H, m), 6.94 (1H, d, J = 8.0 Hz), 7.17 (1H, d, J = 8.5 Hz), 7.29 (1H, s); ¹³C NMR (CD₃OD, 125 MHz): δ 21.0, 27.2, 27.4, 38.4, 67.5, 109.8, 112.4, 117.6, 121.2, 125.0, 130.0, 136.2, 136.8; LRMS (ES⁺) m/z: 258.0 [M+Na⁺]; HRMS (ES⁺) calcd. for C₁₃H₁₃NO₂Cl [M+H⁺]: 236.0842, found 236.0831.

(R)-6-Chloro-2,3,4,9-tetrahydro-1H-carbazole-2-carboxylic acid (1e). To a solution of (R)-3-oxo-cyclohexane-1-carboxylic acid (104 mg, 0.12 mmol) in glacial acetic acid (0.5 mL), 4-chlorophenylhydrazine hydrochloride (136 mg, 0.76 mmol) was added in glacial acetic acid (1.0 mL) and the resulting suspension heated at reflux overnight. The reaction mixture was cooled, diluted with water (15 mL) and extracted with EtOAc (3 x 15 mL). The combined organic extracts were washed with water (10 mL), brine (10 mL), dried over anhydrous MgSO₄ and concentrated. The resulting residue was purified by silica gel column chromatography (20:80:0.5 to 50:50:0.5 Et₂O/petrol/acetic acid) followed by trituration with petrol to give 1e (31 mg, 17% yield) as a yellow crystalline solid: mp 242–244°C (lit. 249–251°C); ¹H NMR (CD₃OD, 500 MHz): δ 1.86–1.94 (1H, m),
2.25–2.27 (1H, m), 2.60–2.66 (1H, m), 2.73–2.78 (1H, m), 2.80–2.86 (1H, m), 2.91 (2H, br d, J = 7.5 Hz), 6.95 (1H, dd, J = 8.5, 1.8 Hz), 7.17 (1H, d, J = 8.5 Hz), 7.29 (1H, s), 10.2 (1H, br s); 13C NMR (CD3OD, 125 MHz): δ 20.9, 26.5, 27.5, 41.3, 109.2, 112.5, 117.7, 121.5, 125.1, 129.8, 135.7, 136.2, 178.9; LRMS (ES⁻) m/z: 248.0 [M-H⁺]; HRMS (ES⁺) calcd. for C13H11NO2Cl [M+H⁺] 248.0478, found 248.0486; [α]255°289 +47.7 (c 1.02, MeOH).

**[(S)-6-Chloro-2,3,4,9-tetrahydro-1H-carbazole-2-carboxylic acid (1f)]**. The compound was prepared according to the method described for 1e from (S)-3-oxo-cyclohexane-1-carboxylic acid (101 mg, 0.71 mmol) and 4-chlorophenylhydrazine hydrochloride (133.0 mg, 0.74 mmol). 1f (59 mg, 33% yield) was obtained as a yellow crystalline solid: mp 244–246°C (lit⁶ 249–250°C); 1H NMR (CD3OD, 500 MHz): δ 1.87–1.95 (1H, m), 2.25–2.28 (1H, m), 2.61–2.67 (1H, m), 2.74–2.79 (1H, m), 2.81–2.86 (1H, m), 2.95 (2H, d, J = 7.0 Hz), 6.95 (1H, dd, J = 8.5, 1.5 Hz), 7.17 (1H, d, J = 9.0 Hz), 7.29 (1H, d, J = 1.5 Hz), 10.19 (1H, br s); 13C NMR (CD3OD, 125 MHz): δ 20.9, 26.6, 27.5, 41.4, 109.3, 112.6, 117.7, 121.5, 125.2, 129.8, 135.8, 136.3, 178.9; LRMS (ES⁺) m/z: 250.0 [M+H⁺⁺]; HRMS (ASAP⁺) calcd. for C13H13NO2Cl [M+H⁺⁺] 250.0635, found 250.0643; [α]255°289 −45.2 (c 1.02, MeOH).

**[(R)-6-Fluoro-2,3,4,9-tetrahydro-1H-carbazole-2-carboxylic acid (1g)]**. The compound was prepared according to the method described for 1e from (R)-3-oxo-cyclohexane-1-carboxylic acid (127 mg, 0.89 mmol) and 4-fluorophenylhydrazine hydrochloride (122 mg, 0.75 mmol). 1g (51 mg, 29% yield) was obtained as a yellow powder: mp 244–246°C; 1H NMR (CD3OD, 300 MHz): δ 1.87–1.96 (1H, m), 2.24–2.28 (1H, m), 2.58–2.86 (3H, m), 2.95 (2H, d, J = 7.2 Hz), 6.75 (1H, t, J = 9.0 Hz), 6.98 (1H, d, J = 9.9 Hz), 7.14–7.18 (1H, m), 10.09 (1H, br s); 13C NMR (CD3OD, 75 MHz): δ 21.0, 26.6, 27.6, 41.4, 103.1 (d, J = 24.0 Hz), 109.1 (d, J = 26.3 Hz), 109.5 (d, J = 4.6 Hz), 112.0 (d, J = 10.3 Hz), 128.9 (d, J = 10.3 Hz), 134.3, 136.0, 158.8 (d, J = 230.0 Hz), 179.0; LRMS (ES⁻) m/z: 232.0 [M-H⁻]; HRMS (ES⁻) calcd. for C13H11NO2F [M-H⁻] 232.0774, found 232.0769; [α]255°289 +16.2 (c 0.52, MeOH).

**[(R)-6-Iodo-2,3,4,9-tetrahydro-1H-carbazole-2-carboxylic acid (1h)]**. The compound was prepared according to the method described for 1e from (R)-3-oxo-cyclohexane-1-carboxylic acid (84 mg, 0.59 mmol) and 4-iodophenylhydrazine (107 mg, 0.46 mmol). 1h (24 mg, 15% yield) was obtained as a brown powder: mp 202–204°C; 1H NMR (CD3OD, 500 MHz): δ 1.88–1.95 (1H, m), 2.25–2.28 (1H, m), 2.60–2.67 (1H, m), 2.73–2.77 (1H, m), 2.81–2.86 (1H, m), 2.95 (2H, d, J = 7.0 Hz), 7.05 (1H, d, J = 9.0 Hz), 7.25 (1H, d, J = 8.0 Hz), 7.65 (1H, s), 10.22 (1H, br s); 13C NMR (CD3OD, 125 MHz): δ 20.9, 26.5, 27.5, 41.4, 82.2, 108.8, 113.6, 127.3, 129.8, 131.3, 135.2, 136.9, 178.9; LRMS (ES⁻) m/z: 340.0 [M-H⁻]; HRMS (ES⁻) calcd. for C13H11NO2I [M-H⁻] 339.9835, found 339.9830; [α]255°289 −27.7 (c 0.28, MeOH).

**[(R)-6-Bromo-2,3,4,9-tetrahydro-1H-carbazole-2-carboxylic acid (1i)]**. The compound was prepared according to the method described for 1e from (R)-3-oxo-cyclohexane-1-carboxylic acid (568 mg, 3.99 mmol) and 4-bromophenylhydrazine hydrochloride (908 mg, 4.06 mmol). 1i (943 mg, 80% yield) was obtained as a yellow powder: mp 230–232°C; 1H NMR (CD3OD, 500 MHz): δ 1.87–1.95 (1H, m), 2.25–2.28 (1H, m), 2.61–2.67 (1H, m), 2.74–2.87 (2H, m), 2.96 (2H, d, J =
7.0 Hz), 7.08 (1H, d, J = 8.5 Hz), 7.14 (1H, d, J = 8.5 Hz), 7.45 (1H, s); $^{13}$C NMR (CD$_3$OD, 125 MHz): δ 20.9, 26.5, 27.5, 41.4, 109.1, 112.6, 113.0, 120.9, 124.1, 130.5, 135.6, 136.4, 178.9; LRMS (ES$^-$) m/z: 294.0 [M-H]$^-$; HRMS (ES$^-$) calcd. for C$_{13}$H$_{11}$NO$_2$Br [M-H]$^-$ 291.9973, found 291.9964; $[\alpha]_{589}^{25}$ +49.0 (c 0.51, MeOH).

(S)-6-Bromo-2,3,4,9-tetrahydro-1H-carbazole-2-carboxylic acid (1j). The compound was prepared according to the method described for 1e from (S)-3-oxo-cyclohexane-1-carboxylic acid (241 mg, 1.70 mmol) and 4-bromophenylhydrazine hydrochloride (405 mg, 1.81 mmol). 1j (212 mg, 42% yield) was obtained as a yellow powder: mp 236–238$^\circ$C; $^1$H NMR (CD$_3$OD, 500 MHz): δ 1.89–1.90 (1H, m), 2.24–2.26 (1H, m), 2.61–2.67 (1H, m), 2.72–2.95 (4H, m), 7.07 (1H, br d, J = 8.0 Hz), 7.13 (1H, d, J = 7.5 Hz), 7.44 (1H, s); $^{13}$C NMR (CD$_3$OD, 125MHz): δ 20.9, 26.5, 27.5, 41.4, 109.1, 112.5, 113.0, 120.9, 124.1, 130.4, 135.6, 136.4, 178.9; LRMS (ES$^-$) m/z: 292.0 [M-H]$^-$; HRMS (ES$^-$) calcd. for C$_{13}$H$_{11}$NO$_2$Br [M-H]$^-$ 291.9973, found 291.9980; $[\alpha]_{589}^{25}$ –39.6 (c 0.52, MeOH).

Ethyl (R)-6-chloro-2,3,4,9-tetrahydro-1H-carbazole-2-carboxylate (1k). To a solution of 4-chlorophenylhydrazine hydrochloride (974 mg, 6.85 mmol) in glacial acetic acid (8 mL) was added (R)-3-oxo-1-cyclohexane carboxylic acid (702 mg, 4.94 mmol) and the suspension heated at reflux for 6 h. The reaction mixture was cooled, diluted with water and extracted with EtOAc (3 x 50 mL). The combined organic extracts were washed with water (50 mL), brine (50 mL), dried over anhydrous MgSO$_4$ and concentrated. The resulting crude residue was redissolved in absolute ethanol (5 mL) containing concentrated H$_2$SO$_4$ (100 µL) and the reaction heated at reflux overnight. The solution was cooled, concentrated, made alkaline with saturated NaHCO$_3$aq and the mixture extracted with EtOAc (3 x 40 mL). The combined organic fractions were washed with brine (40 mL), dried over anhydrous MgSO$_4$ and concentrated. The resulting residue was purified by silica gel column chromatography (10:0–8:2 Et$_2$O/petrol) to give 1k (877 mg, 64% yield) as a yellow powder: mp 130–132$^\circ$C; $^1$H NMR (CDCl$_3$, 500 MHz): δ 1.29 (3H, t, J = 7.3 Hz), 1.91–1.98 (1H, m), 2.28–2.31 (1H, m), 2.64–2.70 (1H, m), 2.77–3.05 (4H, m), 4.16–4.21 (2H, m), 7.06 (1H, d, J = 8.0 Hz), 7.16 (1H, d, J = 8.5 Hz), 7.39 (1H, s), 7.83 (1H, br s); $^{13}$C NMR (CDCl$_3$, 125 MHz): δ 14.4, 20.1, 25.1, 25.7, 26.3, 40.3, 60.9, 109.5, 111.5, 117.6, 121.5, 125.1, 128.6, 134.0, 134.4, 175.1; LRMS (ES$^+$) m/z: 278.0 [M+H]$^+$; HRMS (ES$^+$) calcd. for C$_{15}$H$_{17}$NO$_2$Cl [M+H]$^+$ 278.0948, found 278.0938; $[\alpha]_{589}^{25}$ +48.4 (c 1.05, MeOH).

Ethyl (S)-6-chloro-2,3,4,9-tetrahydro-1H-carbazole-2-carboxylate (1l). The compound was prepared according to the method described for 1k from (S)-3-oxocyclohexane-1-carboxylic acid (272 mg, 1.91 mmol) and 4-chlorophenylhydrazine hydrochloride (340 mg, 1.89 mmol). 1l was obtained (184 mg, 35% yield) as a pale yellow solid: mp 130–132$^\circ$C; $^1$H NMR (CDCl$_3$, 500 MHz): δ 1.29 (3H, t, J = 7.3 Hz), 1.92–1.98 (1H, m), 2.29–2.31 (1H, m), 2.65–2.71 (1H, m), 2.77–3.05 (4H, m), 4.17–4.23 (2H, m), 7.06 (1H, dd, J = 8.0, 1.5 Hz), 7.17 (1H, d, J = 9.0 Hz), 7.40 (1H, s), 7.81 (1H, br s); $^{13}$C NMR (CDCl$_3$, 125 MHz): δ 14.4, 20.1, 25.7, 26.3, 40.3, 60.9, 109.5, 111.5, 117.6, 121.6, 125.1, 128.6, 134.0, 134.4, 175.1; LRMS (ES$^+$) m/z: 276.0 [M+H]$^+$; HRMS (ES$^+$) calcd. for C$_{15}$H$_{15}$NO$_2$Cl [M+H]$^+$ 276.0791, found 276.0780; $[\alpha]_{589}^{25}$ –42.8 (c 1.03, MeOH).

Ethyl (R)-6-bromo-2,3,4,9-tetrahydro-1H-carbazole-2-carboxylate (1m). The compound was prepared according to the method described for 1k from (R)-3-oxocyclohexane-1-carboxylic acid (2.28 g, 10.18
mmol) and 4-bromophenylhydrazine hydrochloride (1.45 g, 10.17 mmol). 1m (1.99 g, 58% yield) was obtained as a yellow powder: mp 144–146°C; $^1$H NMR (CDCl$_3$, 500 MHz): δ 1.29 (3H, t, J = 7.0 Hz), 1.92–1.98 (1H, m), 2.28–2.31 (1H, m), 2.65–2.70 (1H, m), 2.77–3.05 (4H, m), 4.17–4.23 (2H, m, CH$_2$), 7.11 (1H, d, J = 8.5 Hz), 7.18 (1H, d, J = 8.5 Hz), 7.55 (1H, s), 7.84 (1H, br s); $^{13}$C NMR (CDCl$_3$, 125 MHz): δ 14.5, 20.1, 25.7, 26.4, 40.3, 61.0, 109.5, 112.1, 112.7, 120.7, 124.2, 129.3, 133.9, 134.8, 175.2; LRMS (ES$^+$) m/z: 362.0 [M+K$^+$]; HRMS (ES$^+$) calcd. for C$_{15}$H$_7$NO$_2$Br [M+H]$^+$ 322.0443, found 322.0443; [α]$_{589}^2$ +49.5 (c 1.04, MeOH).

Ethyl (S)-6-bromo-2,3,4,9-tetrahydro-1H-carbazole-2-carboxylate (1n). The compound was prepared according to the method described for 1k from (S)-3-oxocyclohexane-1-carboxylic acid (227 mg, 1.60 mmol) and 4-bromophenylhydrazine hydrochloride (387 mg, 1.73 mmol). 1n (183 mg, 35% yield) was obtained as a yellow powder: mp 136–138°C; $^1$H NMR (CDCl$_3$, 500 MHz): δ 1.29 (3H, d, J = 7.3 Hz), 1.90–1.98 (1H, m), 2.28–2.31 (1H, m), 2.64–2.70 (1H, m), 2.76–3.04 (4H, m), 4.16–4.23 (2H, m, CH$_2$), 7.11 (1H, d, J = 8.5 Hz), 7.18 (1H, dd, J = 8.0, 1.5 Hz), 7.55 (1H, s), 7.84 (1H, br s); $^{13}$C NMR (CDCl$_3$, 125 MHz): δ 14.4, 20.1, 25.6, 26.3, 40.3, 60.9, 109.4, 112.0, 112.6, 120.7, 124.1, 129.3, 133.8, 134.7, 175.1; LRMS (ES$^+$) m/z: 320.0 [M–H$^-$]; HRMS (ES$^+$) calcd. for C$_{15}$H$_{15}$NO$_2$Br [M–H$^-$] 320.0286, found 320.0288; [α]$_{589}^2$ –39.3 (c 0.98, MeOH).

(R)-9-(2-Amino-2-oxoethyl)-6-chloro-2,3,4,9-tetrahydro-1H-carbazole-2-carboxylic acid (2a). To a solution of 1k (163 mg, 0.59 mmol) in dry DMF (1.0 mL) was added Cs$_2$CO$_3$ (304 mg, 0.93 mmol) and the suspension stirred at room temperature for 15 min. A solution of iodoacetamide (152 mg, 0.82 mmol) in dry DMF (0.5 mL) was added drop wise and the mixture stirred at 60°C under N$_2$ overnight. The reaction was quenched with 1 M HCl (20 mL) and extracted with EtOAc (3 x 20 mL). The combined extracts were washed with 0.5 M HCl (2 x 20 mL), brine (20 mL), dried over anhydrous MgSO$_4$ and concentrated. The crude residue was purified by silica gel column chromatography (6:4:0–9:0:1 Et$_2$O/petrol/MeOH) to give ethyl (R)-9-(2-amino-2-oxoethyl)-6-chloro-2,3,4,9-tetrahydro-1H-carbazole-2-carboxylate as a yellow powder. The compound was dissolved in absolute EtOH (1 mL) and an aqueous solution of NaOH (2 M, 400 μL) added. The resulting suspension was stirred at room temperature overnight. The reaction mixture was concentrated, diluted with water and washed with CH$_2$Cl$_2$ (10 mL). The aqueous layer was acidified with 1 M HCl and extracted with EtOAc (3 x 10 mL). The combined organic extracts were washed with water (10 mL), brine (10 mL), dried over anhydrous MgSO$_4$ and concentrated. The resulting residue was triturated with EtO to give 2a (11 mg, 6% yield) as a white solid: mp > 260°C (dec.); $^1$H NMR (DMSO-d$_6$, 500 MHz): δ 1.74–1.76 (1H, m), 2.17–2.20 (1H, m), 2.60–2.63 (1H, m), 2.73–2.92 (4H, m), 4.68 (2H, s), 7.05 (1H, d, J = 9.0 Hz), 7.23 (1H, br s), 7.33 (1H, d, J = 8.5 Hz), 7.41 (1H, s), 7.53 (1H, br s); $^{13}$C NMR (DMSO-d$_6$, 125 MHz): δ 19.7, 24.0, 25.6, 39.40, 45.2, 107.9, 110.5, 116.7, 120.1, 123.3, 127.7, 135.4, 136.7, 169.4, 176.0; LRMS (ES$^+$) m/z: 329.0 [M+Na$^+$]; HRMS (ES$^+$) calcd. for C$_{15}$H$_{16}$NO$_2$Cl [M+H]$^+$ 307.0849, found 307.0847; [α]$_{589}^2$ +10.4 (c 0.6, DMSO).

(S)-9-(2-Amino-2-oxoethyl)-6-chloro-2,3,4,9-tetrahydro-1H-carbazole-2-carboxylic acid (2b). The compound was prepared according to the method described for 2a from 1l (170 mg, 0.61 mmol), iodoacetamide (104 mg, 0.56 mmol) and Cs$_2$CO$_3$ (260 mg, 0.80 mmol) in dry DMF (1.5 mL). Following ethyl ester hydrolysis, 2b (7.4 mg, 4% yield) was obtained as a white powder: mp >
260°C (dec.); $^1$H NMR (DMSO-$d_6$, 500 MHz): $\delta$ 1.74–1.76 (1H, m), 2.18–2.20 (1H, m), 2.61–2.62 (1H, m), 2.77–2.91 (4H, m), 4.68 (2H, s, CH$_2$), 7.05 (1H, d, $J$ = 8.5 Hz), 7.23 (1H, br s), 7.34 (1H, d, $J$ = 8.0 Hz), 7.40 (1H, s), 7.53 (1H, br s); $^{13}$C NMR (DMSO-$d_6$, 125 MHz): $\delta$ 19.7, 24.0, 25.7, 39.5, 45.2, 107.9, 110.6, 116.7, 120.1, 123.3, 127.7, 135.4, 136.7, 169.5, 176.0; LRMS (ES$^+$) m/z: 329.0 [M+Na$^+$]; HRMS (ES$^+$) calcd. for C$_{15}$H$_{15}$N$_2$O$_3$ClNa [M+Na$^+$] 329.0669, found 329.0683; $[\alpha]_25^\circ$ = −9.2 (c 0.29, DMSO).

(R)-2-(6-Bromo-2-(ethoxycarbonyl)-1,2,3,4-tetrahydro-9H-carbazol-9-yl)acetic acid (3a). To a solution of 1m (642 mg, 2.31 mmol) in dry DMF (1 mL) was added Cs$_2$CO$_3$ (581 mg, 1.78 mmol) and tert-butyl bromoacetate (302 mg, 1.55 mmol) and the mixture was stirred at 60°C under N$_2$ for 19 h. The reaction mixture was quenched with 1 M HCl (20 mL) and extracted with CH$_2$Cl$_2$ (3 x 20 mL). The combined extracts were washed with 0.5 M HCl (3 x 20 mL), brine (20 mL), dried over anhydrous MgSO$_4$ and concentrated. The resulting crude residue was purified by silica gel column chromatography (2:8–5:5 Et$_2$O/petrol) to give (R)-9-(2-(tert-butoxy)-2-oxoethyl)-6-chloro-2,3,4,9-tetrahydro-1H-carbazole-2-carboxylic acid, which was treated with TFA (1 mL). Following reaction for 1 h, the TFA was evaporated under a stream of N$_2$ to afford 3a (402 mg, 83% yield) as a yellow powder: mp 180–182°C; $^1$H NMR (DMSO-$d_6$, 500 MHz): $\delta$ 1.22 (3H, t, $J$ = 6.8 Hz, CH$_3$), 1.77–1.78 (1H, m), 2.17–2.19 (1H, m), 2.63–2.94 (5H, m), 4.11–4.15 (2H, m), 4.92 (2H, s), 7.17 (1H, d, $J$ = 8.0), 7.35 (1H, d, $J$ = 8.5 Hz), 7.55 (1H, br s), 13.09 (1H, br s); $^{13}$C NMR (DMSO-$d_6$, 125 MHz): $\delta$ 14.1, 19.4, 23.7, 25.6, 39.3, 44.1, 60.2, 108.1, 110.7, 116.8, 120.3, 123.5, 127.7, 135.3, 136.1, 170.3, 174.2; LRMS (ES$^+$) m/z: 380.0 [M-H$^-$]; HRMS (ES$^+$) calcd. for C$_{17}$H$_{17}$NO$_4$Br 378.0341 [M-H$^-$], found 378.0324; $[\alpha]_25^\circ$ +25.7 (c 1.00, MeOH).

2-(6-Chloro-2-(ethoxycarbonyl)-3,4-dihydro-1H-carbazol-9(2H)-yl)acetic acid (3c). The compound was prepared according to the method described for 3a from 1k (642 mg, 2.31 mmol), tert-butyl bromoacetate (375 µL, 495 mg, 2.54 mmol) and Cs$_2$CO$_3$ (1.03 g, 3.15 mmol) in DMF (4 mL). Following tert-butyl ester deprotection 3c (331 mg, 43% yield) was obtained as an pale yellow powder: mp 180–182°C; $^1$H NMR (DMSO-$d_6$, 500 MHz): $\delta$ 1.22 (3H, t, $J$ = 7.0 Hz), 1.75–1.80 (1H, m), 2.17–2.19 (1H, m), 2.60–2.65 (1H, m), 2.73–2.94 (4H, m), 4.13 (2H, t, $J$ = 6.8 Hz), 4.92 (2H, d, $J$ = 2.0 Hz), 7.05 (1H, dd, $J$ = 8.5, 1.8 Hz), 7.39 (1H, d, $J$ = 8.5 Hz), 7.41 (1H, d, $J$ = 2.0 Hz), 13.00 (1H, br s); $^{13}$C NMR (DMSO-$d_6$, 125 MHz): $\delta$ 14.1, 19.5, 23.7, 25.6, 39.3, 44.1, 60.2, 108.1, 110.7, 116.8, 120.3, 123.5, 127.7, 135.3, 136.1, 170.3, 174.2; LRMS (ES$^+$) m/z: 358.0 [M+Na$^+$]; HRMS (ES$^+$) calcd. for C$_{17}$H$_{16}$NO$_4$ClNa [M+Na$^+$] 358.0822, found 358.0815; $[\alpha]_25^\circ$ +2.0 (c 0.97, MeOH).

(R)-9-(2-Amino-2-oxoethyl)-6-bromo-2,3,4,9-tetrahydro-1H-carbazole-2-carboxylic acid (4a). To a solution of 3a (87.9 mg, 0.23 mmol) in dry CH$_2$Cl$_2$ (2 mL) was added NHS (41 mg, 0.36 mmol) and EDC (79 mg, 0.41 mmol) and the reaction mixture stirred for 1 h at room temperature. The CH$_2$Cl$_2$ was evaporated under nitrogen and the resulting residue redissolved in THF (2 mL). Aqueous NH$_4$OH solution (28%; 1 mL) was added and the mixture stirred overnight at room temperature. The resulting precipitate was collected by vacuum filtration (washing with water) and then triturated with EtO. The resulting (R)-9-(2-amino-2-oxoethyl)-6-bromo-2,3,4,9-tetrahydro-1H-carbazole-2-carboxylic acid (29 mg, 0.08 mmol) was dissolved in absolute EtOH (2 mL), an aqueous NaOH solution (2 M; 500 µL) added and the suspension stirred at room temperature overnight. The reaction mixture was concentrated, diluted with water, acidified
with 1 M HCl and extracted with EtOAc (3 x 10 mL). The combined extracts were washed with water (10 mL), brine (10 mL), dried over anhydrous MgSO₄ and concentrated. The resulting crude residue was triturated with Et₂O to give 4a (12 mg, 14% yield) as a yellow powder: mp > 250°C (dec.); ¹H NMR (DMSO-d₆, 300 MHz): δ 1.70–1.76 (1H, m), 2.16–2.20 (1H, m), 2.60–2.92 (5H, m), 4.69 (2H, s), 7.16 (1H, d, J = 8.7 Hz), 7.25 (1H, br s), 7.29 (1H, d, J = 8.7 Hz), 7.54 (2H, br s), 12.44 (1H, br s); ¹³C NMR (DMSO-d₆, 75 MHz): δ 19.7, 23.9, 25.6, 39.3, 45.2, 107.8, 111.1, 111.2, 119.7, 122.7, 128.4, 135.6, 136.5, 169.4, 176.0; LRMS (ES⁺) m/z: 373.0 [M+Na⁺]; HRMS (ES⁺) calcd. for C₁₅H₁₅N₂O₃BrNa [M+Na⁺] 373.0164, found 373.0182; [α]₂⁵ş⁰ +12.9 (c 0.64, DMSO).

(S)-9-(2-amino-2-oxoethyl)-6-bromo-2,3,4,9-tetrahydro-1H-carbazole-2-carboxylic acid (4b). (S)-2-(6-bromo-2-(ethoxycarbonyl)-1,2,3,4-tetrahydro-9H-carbazol-9-yl)acetic acid (3b) was prepared according to the method described for 3a from 1n (145 g, 0.45 mmol), Cs₂CO₃ (220 mg, 0.67 mmol) and tert-butyl bromoacetate (115 mg, 0.59 mmol). Following TFA deprotection of the tert-butyl ester, (S)-2-(6-bromo-2-(ethoxycarbonyl)-1,2,3,4-tetrahydro-9H-carbazol-9-yl)acetic acid (92 mg, 0.24 mmol) was subsequently reacted with NHS (40 mg, 0.34 mmol) and EDC (72 mg, 0.38 mmol) according to the method described for 4a. Ethyl ester hydrolysis in an aqueous solution of NaOH (2 M; 300 µL) in absolute ethanol (4 mL) yielded 4b (28 mg, 33% yield) as a yellow powder: mp > 250°C (dec.); ¹H NMR (DMSO-d₆, 500 MHz): δ 1.75–1.76 (1H, m), 2.17–2.20 (1H, m), 2.59–2.62 (1H, m), 2.73–2.92 (4H, m), 4.69 (2H, s, CH₂), 7.16 (1H, d, J = 8.5 Hz), 7.23 (1H, br s), 7.29 (1H, d, J = 8.5 Hz), 7.52 (1H, br s), 7.54 (1H, s), 12.42 (1H, br s); ¹³C NMR (DMSO-d₆, 125 MHz): δ 19.7, 23.9, 25.6, 39.3, 45.2, 107.8, 111.1, 111.2, 119.7, 122.7, 128.3, 135.6, 136.5, 169.4, 175.9; LRMS (ES⁺) m/z: 373.0 [M+Na⁺]; HRMS (ES⁺) calcd. for C₁₅H₁₅N₂O₃BrNa [M+Na⁺] 373.0164, found 373.0174; [α]₂⁵ş⁰ +4.4 (c 0.71, DMSO).

(R)-9-(2-((carboxymethyl)amino)-2-oxoethyl)-6-chloro-2,3,4,9-tetrahydro-1H-carbazole-2-carboxylic acid (5a). To a solution containing 3c (147 mg, 0.44 mmol), glycine methyl ester hydrochloride (69 mg, 0.55 mmol) and HATU (221 mg, 0.58 mmol) in dry DMF (1 mL) was added DIPEA (250 µL, 1.44 mmol) dropwise and the resulting mixture stirred at room temperature overnight under N₂. The reaction mixture was diluted water and extracted with EtOAc (3 x 15 mL). The combined extracts were washed with 0.5 M HCl (3 x 15 mL), sat. NaHCO₃ (15 mL) and brine (15 mL) before being dried over anhydrous MgSO₄ and concentrated. The resulting residue containing ethyl (R)-6-chloro-9-(2-((2-methoxy-2-oxoethyl)amino)-2-oxoethyl)-2,3,4,9-tetrahydro-1H-carbazole-2-carboxylate was dissolved in absolute EtOH (1 mL), an aqueous solution of NaOH (2 M, 400 µL) added and the suspension stirred at room temperature overnight. The reaction mixture was concentrated, diluted with water and then washed with CH₂Cl₂ (15 mL). The aqueous layer was acidified (1 M HCl) and extracted with EtOAc (3 x 15 mL). The combined organic layers were washed with water (15 mL) and brine (15 mL) before being dried over anhydrous MgSO₄ and concentrated. The resulting residue was purified by silica gel column chromatography (0:10:0–1:9:0.005 MeOH/CH₂Cl₂/acetic acid) to give 5a (96 mg, 60% yield) as an off-white powder: mp 220–222°C; ¹H NMR (CD₃OD, 500 MHz): δ 1.92–1.99 (1H, m), 2.26–2.29 (1H, m), 2.64–2.70 (1H, m), 2.76–2.79 (1H, m), 2.89–2.98 (3H, m), 3.89 (2H, s), 4.77 (2H, s), 7.04 (1H, d, J = 8.5 Hz), 7.23 (1H, d, J = 9.0 Hz), 7.35 (1H, s), 7.96 (1H, br s); ¹³C NMR (CD₃OD, 125 MHz): δ 20.9, 25.2, 27.1, 41.2, 41.8, 46.7, 110.5, 111.0, 118.2, 122.1, 126.1, 129.8, 137.0, 137.2, 171.4, 172.6, 178.6; LRMS (ES⁻) m/z: 363.0 [M-H⁻]; HRMS (ES⁻) calcd. for C₁₇H₁₇N₂O₃Cl [M-H⁻] 363.0748, found 363.0733; [α]₂⁵ş⁰ −5.7 (c 0.50, MeOH).
(R)-6-bromo-9-(2-((carboxymethyl)amino)-2-oxoethyl)-2,3,4,9-tetrahydro-1H-carbazole-2-carboxylic acid (5b). The compound was prepared according to the method described for 5a from 3a (108 mg, 0.28 mmol), glycine methyl ester hydrochloride (49 mg, 0.39 mmol), HATU (137 mg, 0.36 mmol) and DIPEA (160 µL, 0.92 mmol) in dry DMF (1 mL).

Following ester deprotection in an aqueous solution of NaOH (2 M; 300 µL) and absolute ethanol (1 mL), 5b (46 mg, 40% yield) was obtained as an off white powder: mp 238–240°C; \(^1\)H NMR (CD3OD, 500 MHz): \(\delta\) 1.92–1.95 (1H, m), 2.26–2.28 (1H, m), 2.64–2.68 (1H, m), 2.76–2.79 (1H, m), 2.89–2.98 (3H, m), 3.89 (2H, s), 4.76 (2H, s), 7.17–7.18 (2H, m), 7.50 (1H, s), 8.01 (1H, br s); \(^{13}\)C NMR (CD3OD, 125 MHz): \(\delta\) 20.9, 25.1, 27.2, 41.1, 41.8, 46.6, 110.4, 111.5, 113.5, 121.3, 124.7, 130.4, 137.1, 171.3, 172.7, 178.6; LRMS (ES\(^+\)) m/z: 407.0 [M-H\(^-\)]; HRMS (ES\(^+\)) calcd. for C\(_{17}\)H\(_{16}\)N\(_2\)O\(_3\)Br [M-H\(^-\)] 407.0243, found 407.0256; \([\alpha]\)\(_{D}^{20}\) +23.1 (c 0.59, MeOH).

(R)-6-chloro-9-(2-(ethylamino)-2-oxoethyl)-2,3,4,9-tetrahydro-1H-carbazole-2-carboxylic acid (5c). The compound was prepared according to the method described for 5a from 3c (140 mg, 0.42 mmol), ethylamine hydrochloride (46 mg, 0.56 mmol), HATU (211 mg, 0.56 mmol) and DIPEA (200 µL, 1.15 mmol) in dry DMF (1 mL).

Ester deprotection in an aqueous solution of NaOH (2 M; 400 µL) and absolute ethanol (1 mL) afforded 5c (54 mg, 38% yield) as an off-white powder: mp 246–248°C; \(^1\)H NMR (DMSO-d\(_6\), 500 MHz): \(\delta\) 1.02 (3H, t, \(J = 7.0\) Hz), 1.75–1.76 (1H, m), 2.17–2.20 (1H, m), 2.61–2.62 (1H, m), 2.77–2.93 (4H, m), 3.08–3.12 (2H, m), 4.69 (2H, s), 7.05 (1H, d, \(J = 8.0\) Hz), 7.34 (1H, d, \(J = 9.0\) Hz), 7.40 (1H, s), 8.12 (1H, s), 12.43 (1H, br s); \(^{13}\)C NMR (DMSO-d\(_6\), 125 MHz): \(\delta\) 14.6, 19.7, 24.1, 25.6, 33.5, 39.6, 45.6, 108.0, 110.6, 116.7, 120.1, 123.3, 127.7, 135.4, 136.7, 167.0, 176.0; LRMS (ES\(^+\)) m/z: 357.0 [M+Na\(^+\)]; HRMS (ES\(^+\)) calcd. for C\(_{17}\)H\(_{19}\)N\(_2\)O\(_3\)ClNa [M+Na\(^+\)] 357.0982, found 357.0970; \([\alpha]\)\(_{D}^{20}\) +2.7 (c 0.52, MeOH).

(R)-9-(2-(((4-(2-amino-2-oxoethyl)phenyl)amino)-2-oxoethyl)-6-chloro-2,3,4,9-tetrahydro-1H-carbazole-2-carboxylic acid (5d). The compound was prepared according to the method described for 5a from 3c (125 mg, 0.37 mmol), 2-((aminophenyl)acetamide (73 mg, 0.48 mmol), HATU (181 mg, 0.48 mmol) and DIPEA (230 µL, 1.32 mmol) in dry DMF (1 mL).

Ester deprotection in an aqueous solution of NaOH (2 M; 140 µL) and absolute ethanol (1 mL) gave 5d (29 mg, 31% yield) as a white powder: mp > 260°C (dec.); \(^1\)H NMR (DMSO-d\(_6\), 500 MHz): \(\delta\) 1.76–1.78 (1H, m), 2.18–2.20 (1H, m), 2.63–2.96 (5H, m), 3.31 (2H, d, 3.95 (2H, s), 6.82 (2H, s), 7.06 (1H, d, \(J = 7.5\) Hz), 7.19 (2H, d, \(J = 8.0\) Hz), 7.39–7.42 (2H, m), 7.49 (2H, d, \(J = 8.0\) Hz), 10.34 (1H, s), 12.50 (1H, br s); \(^{13}\)C NMR (DMSO-d\(_6\), 125 MHz): \(\delta\) 19.7, 24.0, 25.7, 39.3, 41.7, 46.0, 108.1, 110.6, 116.8, 119.1, 120.2, 123.4, 127.7, 129.4, 131.7, 135.5, 136.8, 136.9, 166.1, 172.2, 176.0; LRMS (ES\(^+\)) m/z: 438.0 [M-H\(^-\)]; HRMS (ES\(^+\)) calcd. for C\(_{23}\)H\(_{21}\)N\(_3\)O\(_4\)Cl [M-H\(^-\)] 438.1221, found 438.1215; \([\alpha]\)\(_{D}^{20}\) +3.77 (c 0.53, DMSO).

(R)-6-bromo-9-(((1-(BOC)piperidin-4-yl)methyl)amino)-2-oxoethyl)-2,3,4,9-tetrahydro-1H-carbazole-2-carboxylic acid (5e). The compound was prepared according to the method described for 5a from 3a (216 mg, 0.57 mmol), tert-butyl 4-(aminomethyl)piperidine-1-carboxylate (159 mg, 0.74 mmol), HATU (301 mg, 0.79 mmol) and DIPEA (350 µL, 2.01 mmol) in dry DMF (2 mL).

Ester deprotection in an aqueous solution of NaOH (2 M; 300 µL) and absolute ethanol (1 mL) provided 5e (68 mg, 36% yield) as a
pale yellow powder: mp 158–160°C; 1H NMR (DMSO-d6, 500 MHz): δ 0.95–0.99 (2H, m), 1.39 (9H, s), 1.57–1.59 (3H, m), 1.74–1.77 (1H, m), 2.17–2.20 (1H, m), 2.61–2.64 (3H, m), 2.73–2.91 (3H, m), 2.94–2.97 (3H, m), 3.91 (2H, br d, J = 9.5 Hz), 4.72 (2H, s), 7.16 (1H, dd, J = 9.0, 1.8 Hz), 7.30 (1H, d, J = 9.0 Hz), 7.54 (1H, d, J = 1.5 Hz), 8.16 (1H, br t, J = 5.8 Hz), 12.44 (1H, br s); 13C NMR (DMSO-d6, 125 MHz): δ 19.7, 24.0, 25.7, 28.1, 29.4, 35.7, 39.4, 44.0, 45.5, 78.5, 107.9, 111.2, 111.3, 119.8, 122.8, 128.4, 135.6, 136.6, 153.8, 167.5, 176.0; LRMS (ES+) m/z: 570.0 [M+Na]+; HRMS (ES+) calcd. for C28H34N3O3BrNa [M+Na]+ 570.1580, found 570.1604; [α]25°$^\text{D}$ +8.5 (c 0.52, MeOH).

(R)-6-bromo-9-(2-oxo-2-((piperidin-4-ylmethyl)amino)ethyl)-2,3,4,9-tetrahydro-1H-carbazole-2-carboxylic acid. TFA salt (5f). Neat TFA (1 mL) was added to 5e (42 mg, 0.076 mmol) and the mixture stirred at room temperature for 1 h. The TFA was evaporated under a stream of N2 and the resulting residue triturated with petrol to give 5f (39 mg, 91% yield) as a yellow powder: mp 212–214°C; 1H NMR (CD3OD, 500 MHz): δ 1.29–1.37 (2H, m), 1.77–1.84 (3H, m), 1.98–1.99 (1H, m), 2.27–2.30 (1H, m), 2.70–2.81 (2H, m), 2.89–2.94 (5H, m), 3.13–3.36 (2H, m), 4.76 (2H, d, J = 3.0 Hz), 7.19 (2H, br s), 7.54 (1H, s); 13C NMR (CD3OD, 125 MHz): δ 20.8, 25.3, 27.1, 27.6, 35.2, 41.0, 44.8, 45.0, 46.8, 110.4, 111.4, 113.5, 121.4, 124.7, 130.5, 137.1, 137.3, 171.0, 178.5; LRMS (ES+) m/z: 448.0 [M+H]+; HRMS (ES+) calcd. for C23H27N3O3Br [M+H]+ 448.1236, found 448.1222; [α]25°$^\text{D}$ +4.6 (c 0.51, MeOH).

(R)-9-(2-((2-amino-oxoethyl)amino)-2-oxoethyl)-6-bromo-2,3,4,9-tetrahydro-1H-carbazole-2-carboxylic acid (5g). The compound was prepared according to the method described for 5a from 3a (122 mg, 0.32 mmol), glycinamide hydrochloride (60 mg, 0.55 mmol), HATU (166 mg, 0.44 mmol) and DIPEA (230 µL, 1.32 mmol) in dry DMF (1 mL). Ester hydrolysis in an aqueous solution of NaOH (2 M; 300 µL) and absolute ethanol (1 mL) afforded 5g (8.5 mg, 6.5% yield) as an off-white powder: mp 232–234°C; 1H NMR (DMSO-d6, 500 MHz): δ 1.74–1.76 (1H, m), 2.17–2.19 (1H, m), 2.61–2.63 (1H, m), 2.73–2.95 (4H, m), 3.66 (2H, d, J = 5.5 Hz, CH2), 4.80 (2H, s, CH2), 7.05 (1H, s), 7.15 (1H, d, J = 8.5 Hz), 7.31–7.35 (2H, m), 7.54 (1H, s), 8.26 (1H, br s), 12.40 (1H, br s); 13C NMR (DMSO-d6, 125 MHz): δ 19.6, 23.9, 25.7, 39.8, 41.8, 45.3, 107.9, 111.2, 119.7, 122.7, 128.4, 135.6, 136.6, 167.7, 170.4, 175.9; LRMS (ES+) m/z: 408.0 [M-H]+; HRMS (ES+) calcd. for C17H12N3O3Br [M-H]– 406.0402, found 406.0404; [α]25°$^\text{D}$ –9.6 (c 0.19, DMSO).

(R)-6-bromo-9-(2-((4-carbamoylbenzyl)amino)-2-oxoethyl)-2,3,4,9-tetrahydro-1H-carbazole-2-carboxylic acid (5h). The compound was prepared according to the method described for 5a from 3a (155 mg, 0.41 mmol), 4-(aminomethyl)benzamide (74 mg, 0.49 mmol), HATU (227 mg, 0.60 mmol) and DIPEA (250 µL, 1.44 mmol) in dry DMF (1 mL). Following ester deprotection in an aqueous solution of NaOH (2 M; 300 µL) and absolute ethanol (1 mL), the mixture was acidified and the resulting precipitate collected by vacuum filtration. The precipitate was basified with 1 M NaOH, dissolved in water and filtered through a 1 cm plug.
of reverse phase silica, washing with water. The aqueous solution was acidified and the precipitate collected by vacuum filtration, washing with ethanol and petrol to give 5h (7.0 mg, 4% yield) as an off-white powder: mp > 230°C (dec); 1H NMR (DMSO-d$_6$, 500 MHz): δ 1.75–1.77 (1H, m), 2.18–2.20 (1H, m), 2.62–2.63 (1H, m), 2.73–2.97 (4H, m), 4.34 (2H, s), 4.83 (2H, s), 7.17 (1H, br s), 7.28–7.38 (4H, m), 7.55 (1H, s), 7.82 (2H, d, J = 7.5 Hz), 7.93 (1H, br s), 8.78 (1H, br s), 12.44 (1H, br s), 13C NMR (DMSO-d$_6$, 125 MHz): δ 19.7, 24.1, 25.7, 40.2, 42.0, 45.6, 108.1, 111.2, 111.4, 119.8, 122.8, 126.9, 127.6, 128.5, 132.9, 135.7, 136.6, 142.5, 167.7, 176.1; LRMS (ES') m/z: 482.0 [M-H]; HRMS (ES') calcd. for C$_{23}$H$_{21}$N$_3$O$_7$Br [M-H]$^-$ 482.0715, found 482.0717; [α]$_{D}^{20}$ +7.4 (c 0.48, DMSO).

(R)-9-((2-(1-(2-amino-2-oxoethyl)piperidin-4-yl)amino)-2-oxoethyl)-6-bromo-2,3,4,9-tetrahydro-1H-carbazole-2-carboxylic acid potassium salt (5i). The compound was prepared according to the method described for 5a from 3a (83 mg, 0.22 mmol), 2-(4-amino-1-piperidinyl) acetamide dihydrochloride (70 mg, 0.25 mmol), HATU (111 mg, 0.29 mmol) and DIPEA (200 µL, 1.15 mmol) in dry DMF (1 mL). Following deprotection in an aqueous solution of KOH (1 M, 38 µL, 0.038 mmol) in THF:EtOH:H$_2$O (1:1:1; 1 mL) the mixture was concentrated, washed with CH$_2$Cl$_2$ (2 x 2 mL) and the aqueous layer lyophilized. The residue was triturated with ether, redissolved in methanol, filtered and concentrated to give the potassium salt of 5i (13 mg, 64% yield) as an off-white gum: 1H NMR (DMSO-d$_6$, 500 MHz): δ 1.47–1.52 (2H, m), 1.68–1.71 (3H, m), 2.06–2.11 (3H, m), 2.27–2.29 (1H, m), 2.48–2.50 (1H, m), 2.65–2.73 (5H, m), 2.81 (2H, s), 3.49–3.51 (1H, m), 4.67 (2H, d, J = 3.5 Hz), 7.09–7.11 (2H, m), 7.16 (1H, br s), 7.25 (1H, d, J = 8.5 Hz), 7.46 (1H, d, J = 2.0 Hz), 8.28 (1H, br d, J = 7.0 Hz); 13C NMR (DMSO-d$_6$, 125 MHz): δ 20.4, 25.6, 27.2, 31.2, 43.0, 45.5, 45.8, 52.1, 61.4, 108.1, 110.9, 119.4, 122.0, 128.8, 135.0, 139.2, 166.7, 171.9, 177.4; LRMS (ES') m/z: 491.0 [M-K+H]$^+$; HRMS (ES') calcd. for C$_{22}$H$_{28}$N$_4$O$_7$Br [M-K+H]$^+$ 491.1294, found 491.1310; [α]$_{D}^{20}$ −4.3 (c 1.28, MeOH).

(R)-6-bromo-9-((R)-(5-((tert-butoxycarbonyl)amino)-1-carboxypentyl)amino)-2-oxoethyl)-2,3,4,9-tetrahydro-1H-carbazole-2-carboxylic acid (5j). The compound was prepared according to the method described for 5a with the following modification. Compound 3a (96 mg, 0.25 mmol) was pre-activated with HATU (136 mg, 0.36 mmol) and DIPEA (160 µL, 0.92 mmol) in dry CH$_2$Cl$_2$ (1 mL) containing a few drops of DMF, before a solution of NH$_2$-(D)-Lys-(BOC)-O'Bu.HCl (107 mg, 0.32 mmol) in dry CH$_2$Cl$_2$ (1 mL) was added. Ester deprotection with LiOH.H$_2$O (27 mg, 0.63 mmol) in THF:EtOH:H$_2$O (1:1:1; 1 mL) yielded 5j (98 mg, 57% yield) as a yellow solid: mp 78–80°C; 1H NMR (DMSO-d$_6$, 500 MHz): δ 1.28 (4H, br s), 1.38 (9H, s), 1.59–1.61 (1H, m), 1.71–1.72 (2H, m), 2.17–2.18 (1H, m), 2.61–2.96 (7H, m), 4.14 (1H, br s), 4.74–4.80 (2H, m), 6.75 (1H, br s), 7.16 (1H, d, J = 8.5 Hz), 7.31 (1H, t), 7.54 (1H, s), 8.48 (1H, t), 12.54 (1H, br s); 13C NMR (DMSO-d$_6$, 75 MHz): δ 19.7, 22.7, 24.0, 25.7, 28.3, 29.1, 30.8, 39.4, 39.8, 45.2, 52.1, 77.4, 107.9, 111.2, 111.3, 119.7, 122.7, 128.4, 135.6, 136.6, 155.6, 167.5, 173.4, 176.0; LRMS (ES') m/z: 580.0 [M-H]$^+$; HRMS (ES') calcd. for C$_{28}$H$_{33}$N$_3$O$_7$Br [M-H]$^-$ 578.1502, found 578.1504; [α]$_{D}^{25}$ +2.4 (c 2.84, MeOH).

(R)-6-bromo-9-((S)-1-carboxy-3-methylbutyl)amino)-2-oxoethyl)-2,3,4,9-tetrahydro-1H-carbazole-2-carboxylic acid (5k). The compound was prepared according to the method described for 5a from 3a
(R)-6-bromo-9-{2-[(R)-1-carboxy-2-phenylethyl]amino}-2-oxoethyl-2,3,4,9-tetrahydro-1H-carbazole-2-carboxylic acid (5l). The compound was prepared according to the method described for 5a from 3a (134 mg, 0.35 mmol), NH₂-(D)-Phe-(OMe).HCl (92 mg, 0.43 mmol), HATU (173 mg, 0.46 mmol) and DIPEA (230 µL, 1.32 mmol) in dry DMF (1 mL). Global ester deprotection in an aqueous solution of NaOH (2 M; 300 µL) and absolute ethanol (2 mL) afforded 5l (69 mg, 39% yield) as an off-white powder: mp 194-196 °C; ¹H NMR (CD₃OD, 500 MHz): δ 1.72-1.73 (1H, m), 2.15 (1H, m), 2.58-2.86 (5H, m), 2.86-2.90 (1H, m), 3.06-3.09 (1H, m), 4.41 (1H, br s), 4.65-4.77 (2H, m), 7.11-7.25 (7H, m), 7.52 (1H, s), 8.36 (1H, br s), 12.70 (1H, br s); ¹³C NMR (DMSO-d₆, 125 MHz): δ 19.6, 23.8, 25.6, 36.7, 45.1, 53.4, 53.5, 107.9, 111.2, 111.3, 1119.7, 122.7.7, 126.5, 128.2, 128.4, 129.1, 135.5, 136.4, 137.3, 167.3, 172.6, 175.9; LRMS (ES⁺) m/z: 522.9 [M+Na]+; HRMS (ES⁺) calcd. for C₂₂H₂₃N₂O₅BrNa [M+Na]+ 521.0688, found 521.0686; [α]_{D}^{25} +22.9 (c 0.52, MeOH).

(R)-6-bromo-9-{2-[(S)-1-carboxy-2-phenylethyl]amino}-2-oxoethyl-2,3,4,9-tetrahydro-1H-carbazole-2-carboxylic acid (5m). The compound was prepared according to the method described for 5a from 3a (129 mg, 0.34 mmol), NH₂-(L)-Phe-(OMe).HCl (84 mg, 0.39 mmol), HATU (163 mg, 0.43 mmol) and DIPEA (200 µL, 1.15 mmol) in dry DMF (1 mL). Global ester deprotection in an aqueous solution of NaOH (2 M; 300 µL) and H₂O:EtOH (1:1; 2 mL) gave 5m (98 mg, 57% yield) as a yellow solid: mp 208–210°C; ¹H NMR (DMSO-d₆, 500 MHz): δ 1.73–1.75 (1H, m), 2.16 (1H, m), 2.59–2.60 (1H, m), 2.70–2.76 (3H, m), 2.87–2.93 (2H, m), 3.08–3.11 (1H, m), 4.47–4.84 (1H, m, CH), 4.66–4.78 (2H, m), 7.12–7.29 (7H, m), 7.53 (1H, s), 8.44–8.46 (1H, m), 12.63 (1H, br s); ¹³C NMR (DMSO-d₆, 125 MHz): δ 19.6, 23.8, 25.7, 36.7, 39.4, 45.1, 53.3, 107.9, 111.2, 111.3, 119.7, 122.7, 126.5, 128.2, 128.4, 129.1, 135.5, 136.5, 137.3, 167.3, 172.6, 175.9; LRMS (ES⁺) m/z: 521.0 [M+Na]+; HRMS (ES⁺) calcd. for C₂₂H₂₃N₂O₅BrNa [M+Na]+ 521.0688, found 521.0710; [α]_{D}^{25} +17.8 (c 0.48, MeOH).
Supplementary References


