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Synthesis and biological evaluation of [D-lysine]⁸cyclosporin A analogs as potential anti-HCV agents

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ABSTRACT

An efficient synthesis of [D-lysine]⁸cyclosporin A has been developed. Several analogs of [D-lysine]⁸cyclosporin A have been synthesized and show promising anti-HCV activity, particularly compounds **39** and **43**, which each exhibit an anti-HCV EC₅₀ <200 nM, and are each ≥50-fold less immunosuppressive than cyclosporin A.

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Nearly 200 million people worldwide are infected with hepatitis C, a disease of the liver caused by the hepatitis C virus (HCV), which can lead to liver fibrosis, liver cirrhosis, liver cancer, and ultimately death.¹ There is presently no vaccine for HCV, and current interferon-based therapeutics are successful in only 40–50% of patients treated,¹ prompting the need for new drugs with alternative modes of action. While there are several drugs in development that target key HCV proteins, most notably NS3–4A serine protease and NS5B RNA-dependent RNA polymerase,^{1,2} there remains a need to find additional drug targets to increase options for drug cocktails that would combat HCV drug resistance.

Cyclosporins are cyclic undecapeptides that exhibit a broad spectrum of biological activities.³ Cyclosporin A (**1**, CsA, shown in Fig. 1), isolated from the fungus *Tolypocladium inflatum*, is a powerful immunosuppressant of T-cells, and is the active ingredient of Sandimmune® and Neoral® for preventing organ transplant rejection, as well as Restatis® for treating the autoimmune disease dry eye.³ CsA exerts its immunosuppressive activity by binding to two proteins sequentially to form a ternary complex.⁴ The first of these is a cyclophilin (Cyp), which is a *cis-trans* proline isomerase, the most predominant in humans being cyclophilin A (CypA).⁵ The binary CsA–CypA complex is a potent inhibitor of the phosphatase activity expressed by calcineurin (CN), a calcium-dependent serine/threonine phosphatase that promotes the synthesis of T-cell lymphokines such as interleukin-2 (IL-2).⁶ Thus, CN inhibition ultimately suppresses immune response.⁶

X-ray crystallography has revealed which residues of CsA bind to CypA and CN, respectively.⁴ Figure 1 shows that residues 9, 10, 11, 1, and 2 form the ‘cyclophilin binding domain’ that binds to CypA, while residues 4–7 comprise the ‘calcineurin binding domain’ that binds to CN. Residues 3 and 8 are at the interfaces between these two binding domains, and can potentially have an impact on both CypA and CN binding. Figure 2 depicts how CsA binds to CypA and CN from X-ray crystallographic data, with key CypA and CN residues that form hydrogen bonds shown.

In addition to the immune diseases discussed above that can be addressed by CN inhibition, there are a variety of diseases that are treated by Cyp inhibition alone, most notably the infectious diseases caused by human immunodeficiency virus 1 (HIV-1)⁷ and HCV.⁸ At the moment, there is some debate regarding what role Cyp plays in HCV inhibition, and in fact which Cyp (CypA or CypB) is the operative agent, although recently a consensus has emerged indicating that CypA is the predominant if not exclusive

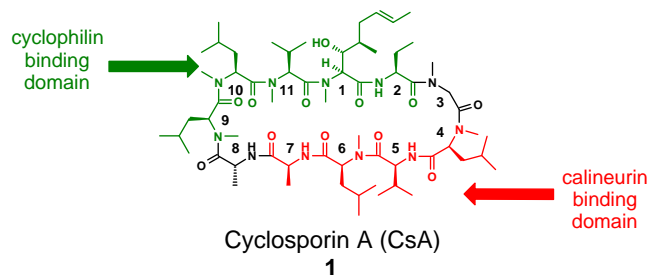


Figure 1. The binding domains of CsA.

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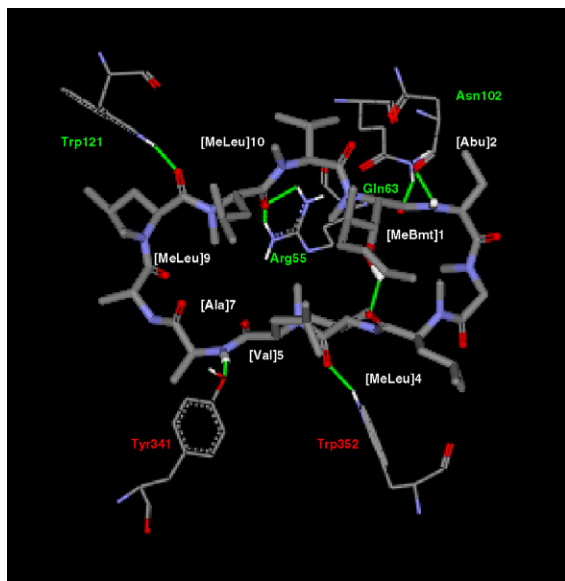


Figure 2. CsA in its active conformation with hydrogen bonds to both CypA and CN. CsA residues are labeled in white; CypA residues are labeled in green; CN residues are labeled in red.

Cyp that supports HCV-specific RNA replication and protein expression in human cells.⁹ In any event, a drug that inhibited a host Cyp protein would clearly complement a drug that targeted a viral protein¹⁰; however, a CsA-based drug used to treat such an infectious disease must be devoid of immunosuppressive activity, as this would antagonize host immune mechanisms for clearing the virus.

Cyclosporins that have been developed for treating HIV and/or HCV are shown in Figure 3. Each of these compounds possess a modification at the 4-position that interferes with CN binding, but not Cyp binding. NIM-811¹¹ (**2**) has an *N*-methylisoleucine at the 4-position instead of an *N*-methylleucine; Debio-025¹² (**3**) has a *D*-alanine at the 3-position and an *N*-ethylvaline at the 4-position; our clinical candidate SCY-635¹³ (**4**) has a dimethylaminoethyl-thioether at the 3-position and a 4'-hydroxymethylleucine at the 4-position.

In our drug discovery program focused on expanding SAR around SCY-635, we were curious if a water-solubilizing amine-bearing tether at the 8-position would have a similar anti-HCV effect as it does at the 3-position of SCY-635. Furthermore, we wondered if lengthening an amine sidechain at position 8 would attenuate CN binding without reducing Cyp binding, as is reported for 8-position modified cyclosporins,¹⁴ particularly [D-Ser]⁸CsA analogs.^{14a,b} In this study, we report a synthesis of [D-lysine]⁸cyclosporin A ([D-Lys]⁸CsA), a known precursor to a reagent used in an ELISA assay to measure a compound's binding affinity for Cyp.¹⁵ We then present the synthesis of several [D-Lys]⁸CsA analogs and evaluate their anti-HCV activity along with their CypA binding, CypB binding, and immunosuppressive activity.

Although there is a published total synthesis of [D-Lys]⁸CsA¹⁶ in 20 steps in an overall yield of 7.0–11.4%, it does not include the synthesis of the tripeptide composing residues 9–11, nor the total synthesis of the (4*R*)-4-[(*E*)-2-butenyl]-4-methyl-L-threonine (MeBmt) residue at the 1-position. The former requires six steps in 68% yield,¹⁷ while the highest yield reported to date for the latter is 22% in 12 steps,¹⁸ bringing the overall yield of [D-Lys]⁸CsA to 1.0–1.6% in a prohibitive 38 steps. In the years since these publications, technology has allowed for the industrial-scale biosynthesis of CsA, which can now serve as a viable starting material. Our synthesis (Scheme 1) starts from CsA and combines regioselective ring-opening methodology first reported by Seebach¹⁹ and further exemplified by Eberle,²⁰ Edman degradation and amino acid coupling chemistry to replace *D*-alanine with *N*-Boc-*D*-lysine at the 8-position,^{20,16} and then ring closure as reported by Rich.¹⁶

CsA (**1**) was first treated with acetic anhydride to protect the hydroxyl group of MeBmt.²¹ The resulting acetate (**5**) was then treated with 1.0 equiv of Lawesson's reagent, which regioselectively reacted with the amide carbonyl groups of residues 4 and 7 to give a statistical mixture of 7-thioamide (**6**), 4-thioamide (**7**), 4,7-bisthioamide (**8**), and unreacted **5**, all of which were partially separated by chromatography, and identified by reported proton NMR spectra.^{19,20} A mixture of **6** and **8** was then treated with benzyl bromide and DBU to give the corresponding benzyl thioimidates.¹⁷ The 4,7-bisbenzyl thioimide (**10**) was considerably less polar than the desired 7-benzyl thioimide (**9**), and the components of the resulting mixture were more readily separated by chromatography than then the corresponding mixture of thioamides **6** and **8**. Hydrolysis of **9** with HCl afforded 7,8-secocyclosporins **11**, which was then subjected to Edman degradation conditions to first prepare thiourea **12** and then decapeptide **13**.²⁰ Installation of Fmoc-protected *D*-Lys and related *D*-amino acids afforded undecapeptides **14–19**.¹⁶ Treatment with base hydrolyzed the acetate at [MeBmt]¹, the thioester at [Ala]⁷, and the Fmoc group of the 8-position to give 7,8-secocyclosporin **20–25**.¹⁶ Cyclization with *n*-propylphosphonic anhydride under dilute conditions gave cyclic undecapeptides **26–31**¹⁶; in the case of amides **24** and **25**, cyclization occurred with concomitant amide dehydration to give nitriles **30** and **31**. Subsequent treatment of Boc-protected amino acids **26–29** with CF₃CO₂H yielded primary amines **32–35**,¹⁶ including [D-Lys]⁸CsA (**35**), which was prepared in 6.3% overall yield in 10 steps, and compares favorably to the previously reported 1.0–1.6% overall yield in 38 steps. We then treated primary amines **32–35** with formaldehyde or acetaldehyde under reductive amination conditions to ultimately afford dimethylamines **36–39** and diethylamines **40–43**, respectively.

Biological activity of [D-Lys]⁸CsA analogs is presented in Table 1. Compound binding affinity to CypA and CypB was measured using an ELISA assay, as described by Quesniaux.¹⁵ In most cases, binding to CypA was within fivefold of that of CsA and SCY-635 themselves, although there were exceptions (**26**, **32**, **33**, **34**, **37**, **40**, **41**) which showed weaker Cyp binding affinity. For most compounds, CypB binding was within twofold of that of CypA binding; however, two of the four compounds with a lengthy 4-carbon

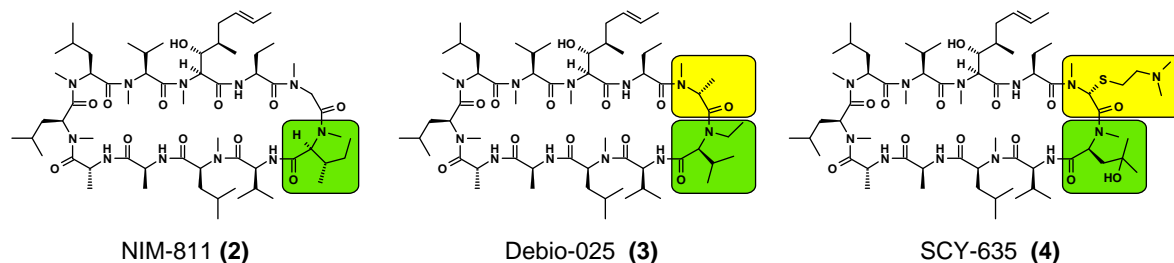
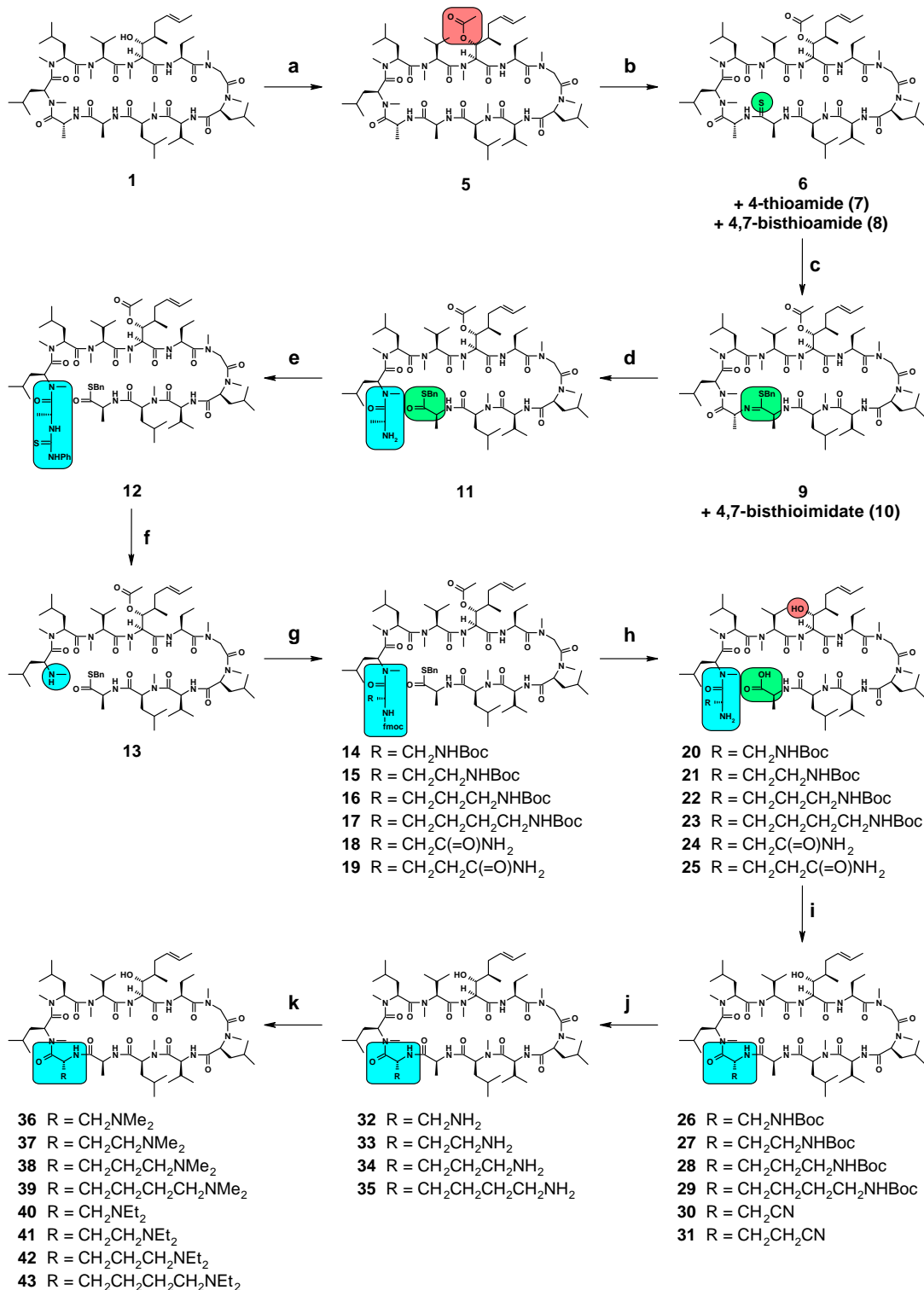


Figure 3. Non-immunosuppressive cyclosporins.

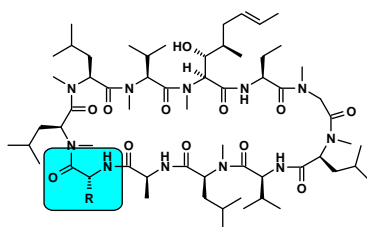


Scheme 1. Reagents and conditions: (a) Ac₂O, DMAP, rt, 12 h, 95%; (b) Lawesson's reagent, *o*-xylene, 130 °C, 30 min, 25% (15% as a mixture with 4,7-bisthioamide; 10% as a mixture with 4-thioamide and unreacted **5**); (c) BnBr, DBU, CH₂Cl₂, rt, 1 h, 74%; (d) 6 N HCl, CH₃CN, rt, 1 h; (e) PhNCS, CH₃CN, rt, 2.5 h, 86% (two steps); (f) 6 N HCl, CH₃CN, rt, 4 h, 87%; (g) Fmoc-amino acid, EDC-HCl, CH₂Cl₂, rt, 18 h, 89–99%; (h) 2 N NaOH, CH₃OH, rt, 2.5 h; (i) (*n*-PrO₂)₃, DMAP, 0.2 mM in CH₂Cl₂, rt, 60 h, 37–54% (two steps); (j) CF₃CO₂H, CH₂Cl₂, 0 °C to rt, 45 min, 73–88%; (k) NaB(OAc)₃H, H₂C=O in CH₃OH or CH₃C(=O)H in CH₂Cl₂, rt, 24 h, 80–90%.

chain at the 8-position (**34** and **39**) exhibited distinctly greater binding to CypA than CypB, while the other two compounds of this series (**29** and **43**) showed comparable affinity to both CypA and CypB. A possible explanation for this difference in CypA and CypB binding is that a key Cyp loop containing an Arg (Arg¹⁴⁸ in CypA; Arg¹⁵⁸ in CypB) proximal to the CsA 8-residue is positioned

differently in CypA than it is in CypB.²² X-ray crystal structures of CypA–CsA and CypB–CsA complexes suggest that the CsA 8-position α -carbon is closer to this particular Arg guanidine moiety in CypA (~6.5 Å) than it is in CypB (~15.0 Å). This may in turn introduce a distinction between CypA and CypB binding in those CsA analogs with lengthier 8-position substituents. Beyond this, there

Table 1
Biological activity of [D-Lys]⁸CsA analogs



Compound	R	ELISA CypA IC ₅₀ (nM)	ELISA CypB IC ₅₀ (nM)	IL-2 EC ₅₀ (nM)	HCV EC ₅₀ /EC ₉₀ (nM)	Huh-7 cytotoxicity IC ₅₀ /IC ₉₀ (nM)
26	CH ₂ NHBoc	83.5	66.8	50	200/480	1719/>10,000
27	CH ₂ CH ₂ NHBoc	31.2	26.1	22	140/360	2015/>10,000
28	CH ₂ CH ₂ CH ₂ NHBoc	26.7	30.5	21	120/310	2069/>10,000
29	CH ₂ CH ₂ CH ₂ CH ₂ NHBoc	22.8	20.6	121	160/300	2565/>10,000
30	CH ₂ C≡N	9.12	9.45	5.1	40/140	2818/>10,000
31	CH ₂ CH ₂ C≡N	23.0	24.2	12	100/270	2371/>10,000
32	CH ₂ NH ₂	103	79.7	13	300/820	4612/>10,000
33	CH ₂ CH ₂ NH ₂	218	151	90	660/2350	5303/>10,000
34	CH ₂ CH ₂ CH ₂ NH ₂	662	1583	111	1890/6740	>10,000/>10,000
35	CH ₂ CH ₂ CH ₂ CH ₂ NH ₂	11.1	12.7	94	1000/2850	>10,000/>10,000
36	CH ₂ NMe ₂	22.9	25.2	20	190/670	2054/>10,000
37	CH ₂ CH ₂ NMe ₂	46.0	59.5	24	170/300	3275/>10,000
38	CH ₂ CH ₂ CH ₂ NMe ₂	35.2	28.9	39	140/380	2154/8733
39	CH ₂ CH ₂ CH ₂ CH ₂ NMe ₂	14.0	97.1	332	140/300	2807/>10,000
40	CH ₂ NEt ₂	140	158	55	380/950	2683/>10,000
41	CH ₂ CH ₂ NEt ₂	65.2	55.9	114	170/350	1496/8620
42	CH ₂ CH ₂ CH ₂ NEt ₂	21.4	17.2	134	150/370	1817/8913
43	CH ₂ CH ₂ CH ₂ CH ₂ NEt ₂	26.6	19.8	2168	180/530	2239/7897
1 (CsA)		8.33	8.78	6.7	370/910	5810/18,750
4 (SCY-635)		9.48	10.3	4209	50 ^a /240 ^a	>10,000/>10,000

^a These values were obtained when tested alongside compounds **26–43**.

is no obvious trend suggesting that either chain length or nitrogen basicity has a clear influence on Cyp binding, although primary amines (**32–35**) in three of four cases showed distinctly weaker Cyp binding.

Immunosuppressive activity was determined by an interleukin-2 (IL-2) inhibition assay, as IL-2 is a key product of T-cell stimulation that is mediated by calcineurin activation. The IL-2 assay measures inhibition of IL-2 production in Jurkat cells stimulated with phytohemagglutinin-M and phorbol myristate acetate.²³ As a reference, when SCY-635 (**4**, IL-2 EC₅₀ = 4209 nM) was administered to human clinical trial patients at a dose of 3 × 300 mg per day for 15 days, a subset of four patients analyzed showed no significant (i.e., <10%) reduction in plasma IL-2 concentrations.²⁴ Of the set shown in Table 1, three compounds with relatively small substituents at position 8 (**30–32**) showed immunosuppressive activity rivaling that of CsA; several others were within 10-fold of that of CsA. It is noted that within a given series of compounds with identical N-substituents, immunosuppression tended to attenuate with increasing chain size at position 8. The two least immunosuppressive compounds, **39** and **43**, include a tertiary amine nitrogen five atoms away from the ring followed by one or two carbons, suggesting that a basic chain reaching this size threshold may have a repulsive interaction with CN. Analogs that also possess a nitrogen five atoms away from the ring but are either less basic (**29**) or less bulky (**35**) were more immunosuppressive. While none of these compounds were found to be as non-immunosuppressive as SCY-635 itself, compounds **29**, **34**, **39**, **41**, **42**, and in particular **43** demonstrate that adding length to the 8-position substituent can reduce immunosuppression to within twofold of that of SCY-635.

Anti-HCV activity was assessed in the con **1b** derived bi-cistronic replicon containing a stable luciferase reporter at 72 h.²⁵ Cytotoxicity in Huh-7 cells was determined by measuring the release of lactate dehydrogenase. HCV data reveals one compound (**30**) with an

EC₅₀ <100 nM, and a total of 13 out of 18 with an EC₅₀ ≤ 200 nM. The only sub-series showing weak activity were the primary amines (**32–35**), the latter two of which have an EC₅₀ >1000 nM, possibly due to poor cell permeability. While many of these compounds exhibited anti-HCV activity comparable to that of SCY-635, they were also distinctly more cytotoxic in the Huh-7 assay than SCY-635, which exhibited no cytotoxicity up to 10 μM.

In summary, we have synthesized [D-Lys]⁸CsA in 6.3% yield in 10 steps from CsA. We then synthesized [D-Lys]⁸CsA analogs that show anti-HCV activity greater than or comparable to that of CsA, several of which are significantly less immunosuppressive than CsA. In particular, compounds **39** and **43** each exhibit an anti-HCV EC₅₀ <200 nM, and are each ≥50-fold less immunosuppressive than CsA. While none of these compounds combine the anti-HCV potency, lack of immunosuppression, and safety profile exhibited by SCY-635, these results suggest the 8-position of CsA holds promise as a synthetically feasible site for modification towards potential anti-HCV therapeutics.

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