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Comparative pharmacokinetic profile of cyclosporine (CsA) with a decapeptide and a linear analogue†

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The synthesis and *in vivo* pharmacokinetic profile of an analogue of cyclosporine is disclosed. An acyclic congener was also profiled in *in vitro* assays to compare cell permeability. The compounds possess similar calculated and measured molecular descriptors however have different behaviors in an RRCK assay to assess cell permeability.

Introduction

High throughput screening has become a mainstay of the drug discovery process to provide molecular lead matter for novel targets.[1] The likelihood of success of a screening campaign is dependent upon both the quality of the biological screen and the molecular library used to interrogate the system. Many organisations have invested in expanding the chemical diversity of their screening files to provide competitive advantage. Simultaneously there has been an increased interest in the use of macrocycles as a structural class to address complex targets such as class B GPCRs or protein–protein interactions (PPIs) which could provide breakthrough medicines.[2] It has been postulated that macrocycles could combine the strengths of small molecules with biopharmaceuticals.[3] In order to enrich the Pfizer screening file with macrocycles that have the potential to passively cross cell membranes we initiated a campaign to design and construct a library based around cyclosporine A (CsA). Despite unfavourable molecular descriptors for oral bioavailability such as molecular weight, hydrogen bonding potential and lipophilicity[4] CsA combines the potential for passive transcellular absorption across the gut wall with sufficient metabolic stability in the liver. This enables CsA to display systemic exposure post oral dosing for target engagement. Furthermore, macrocycles with a molecular weight range of approximately 600–1000 Da can interact with protein interfaces of 800–1000 Å^2.[5] Beyond CsA macrocycles have delivered other drugs and clinical assets.[6] For members of the drug discovery community access to macrocyclic compounds for broad screening purposes is imperative. Moreover, prospectively designing the library of macrocycles to be membrane permeable could enable the library compounds to engage and modulate intracellular protein targets.[7] Proactive design of this library expands the utility of the library into phenotypic screens and screens using whole cells.[8] It is an over-simplification to assume that compounds such as CsA cross the gut wall *via* a single mode (paracellular *vs.* transcellular *vs.* active transport) however we assume a significant contribution is from a passive transcellular mode.[9] Other research groups have disclosed research into delivering libraries of cell permeable macrocycles inspired by natural products[10] or based on synthetically accessible scaffolds.[11]

Cyclosporine A (CsA, (**1**), Fig. 1) is a key component of patient treatment post organ transplant surgery, acting as an immunosuppressant.[12] Research groups engaged in total synthesis of naturally occurring molecules have been intrigued by the complex structure of this 11 amino acid macrocycle, which

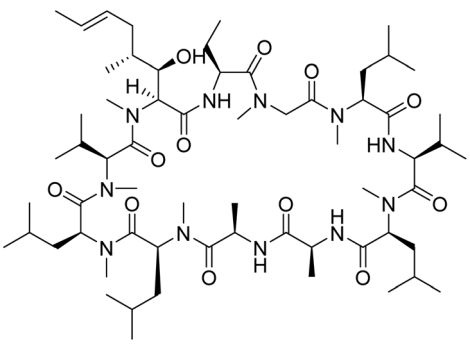

Fig. 1 Cyclosporin A (CsA, **1**).

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upon close inspection reveals a single D-amino acid and multiple sites of *N*-methylation. Indeed, seven of the eleven available amino acids are *N*-methylated, contributing to the hydrophobicity of this peptide. Undoubtedly, CsA has driven the evolution of peptide synthesis in the drug discovery community using both solution and solid phase techniques.[13] In addition, the presence of the unusual amino acid (2*S*,3*R*,4*R*,6*E*)-3-hydroxy-4-methyl-2-methylamino-6-octenoic acid is of importance to the pharmacological activity of CsA. Our strategy was to either completely excise this residue or replace with alternative simpler expressions. The literature suggests this would eliminate or modulate the immune suppressant activity of the analogues.[14] In this publication we disclose a novel cyclic CsA analogue (**2**) and its pharmacokinetic profile post oral dosing in rats. We also disclose the profile of an acyclic analogue (**3**) which was designed to possess as comparable a physicochemical profile to (**2**) as possible. From the *in vitro* profile of (**3**) it is unlikely to have significant oral bioavailability and was not progressed to further animal studies.

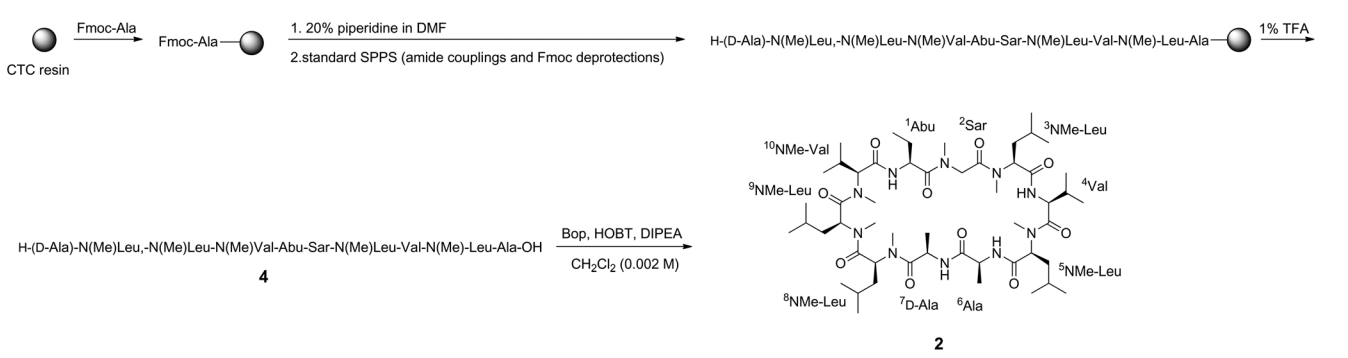
Results and discussion

Synthesis

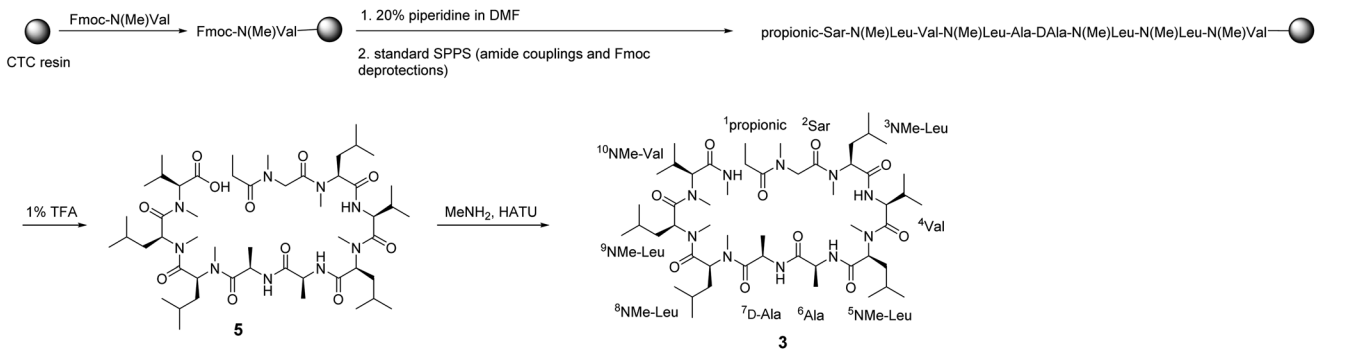
Peptides (**2**) and (**3**) were primarily synthesized using standard Fmoc solid phase peptide synthesis methods using a 2-chlorotritylchloride (CTC) resin. Although details of the syntheses are described in the ESI,† many of the resin based amidations were not straight forward because sterically hindered *N*-methyl-amino termini were involved. Hence, an iterative approach was necessary where a given Fmoc-amino acid, the coupling agent, and base were added multiple times to achieve complete amidation. In the case of peptide (**2**), cleavage of the fully elaborated peptide from the resin afforded peptide (**4**) (Scheme 1). Under dilute solution phase conditions (0.002 M), the peptide underwent a macrolactamization in the presence of Bop and HOBT to deliver cyclic peptide (**2**) in 11% overall yield from the Fmoc-Ala-resin. To afford peptide (**3**), the penultimate intermediate (**5**) was obtained *via* mild TFA cleavage from the resin. Solution phase amidation of (**5**) with methylamine in the presence of HATU delivered the target peptide in 3% overall yield from the Fmoc-N(Me)Val-resin (Scheme 2). Based on HPLC analysis, there was no evidence of Ala epimerization of peptide **4** or the macrocyclization step to deliver peptide **2**.

Pharmacokinetics of CsA and analogues

The passive permeabilities (P_{app}) of (**2**) and CsA were examined side by side in a cell-based permeability assay that uses Ralph Russ canine kidney (RRCK) cells derived from the Madin–Darby canine kidney (MDCK) cell line.[15] RRCK cells were selected by flow cytometry to have little or no functional



Scheme 1 Synthesis of peptide (**2**).



Scheme 2 Synthesis of peptide (**3**).

P-glycoprotein and therefore provide a good tool for measuring passive, transcellular permeability with minimal interference from active-transport mechanisms associated with *P*-glycoprotein expression. Macrocycle (**2**) and CsA showed virtually identical P_{app} values (Table 1) in RRCK cells (pH 6.5/7.4), which are consistent with moderate oral absorption *via* the transcellular route.[16] For both (**2**) and CsA there was good recovery of material (>80%) in the apical to basolateral direction of the assay.

Because of its favorable cell permeability, the *in vivo* pharmacokinetics of (**2**) were examined in male Wistar-Han rats after intravenous (i.v.) and oral (p.o.) dosing (Table 2). The i.v. pharmacokinetics of (**2**) in rats are characterized by a low plasma clearance (CL_p) (9.8 mL min^{-1} kg^{-1}) and a moderate volume of distribution (Vd_{ss}) (0.92 L kg^{-1}), leading to a terminal elimination half-life ($t_{1/2}$) of 2.9 h. The absolute bioavailability (F) of (**2**) in rats after p.o. administration was determined to be ~17%. The fraction of the oral dose absorbed (F_a) was estimated using the equation: $F_a = F/(1 - CL_p/Q)$. Using the rat hepatic blood flow of 70 mL min^{-1} kg^{-1}, F_a was estimated to be 20% for (**2**). Overall, the pharmacokinetics of **2** were comparable to the parameters previously reported for CsA in rats after i.v. and p.o. administration.[16] The slightly higher oral F (~29%) and F_a (~30%) for CsA is consistent with its lower CL_p relative to (**2**) in rats. Corresponding incubations of CsA and (**2**) (1 μM, each) in NADPH-supplemented rat liver microsomes revealed that (**2**) was considerably more unstable ($t_{1/2}$ = 49 min; apparent intrinsic clearance (CL_{int}) = 14.3 μL min^{-1} mg^{-1}) than CsA ($t_{1/2}$ > 120 min; apparent intrinsic clearance (CL_{int}) < 6.0 μL min^{-1} mg^{-1}). The observation that (**2**) was resistant towards metabolic turnover ($t_{1/2}$ > 120 min; apparent intrinsic clearance (CL_{int}) < 6.0 μL min^{-1} mg^{-1}) in microsomal incubations that lacked NADPH cofactor suggested a role for a rat cytochrome P450 enzyme(s) in the oxidative metabolism of (**2**) as a cause of metabolic instability. Incidentally, both CsA and (**2**) were stable ($t_{1/2}$ > 120 min) in incubations with fresh rat plasma, which ruled out the possibility of proteolytic cleavage as a causative factor for the relatively low absorption of the two compounds in the rat. As such, the human plasma/serum stability of CsA has been previously established.[17]

At the outset, this exploratory work was to support the design of a library of cell permeable compounds inspired by CsA for broad screening purposes in Pfizer. During the course of the project a cross comparison of the pharmacokinetic profiles of the cyclic analogue (**2**) with the acyclic (**3**) provided further evidence of the value of cyclisation as a key strategy in modulating the cell permeability of peptides. Designing experiments to compare the cell and membrane permeability of cyclic *versus* acyclic peptides and peptidomimetics are challenging as cyclisation often alters the overall physicochemical profile thus making the interpretation of the data difficult with inter-dependant variables. A seminal publication compared linear and cyclic hexapeptides in the molecular weight (MW) range of 556–629 Da and concluded cyclic peptides have increased permeability due to an increase in lipophilicity. The increase in lipophilicity of these cyclic analogues was suggested to be due to secondary structure involving two beta turns masking hydrogen bonding capabilities.[18] Since this publication there has been an evolution in both calculated and experimental molecular descriptors such as EPSA.[19] For such Beyond Rule of Five molecules,[20] property guidelines for permeability should be computed from their optimal 3-D conformations. A recent analysis showed that radius of gyration (R_{gyr}) and 3D-polar surface area (3D-PSA) are better discriminators of permeability than MW and topological polar surface area (TPSA) for compounds with MW > 550 Da; with enhancements in permeability achieved for molecules when the radius of gyration is ≤7 Å and 3D-PSA is ≤100 $Å^2$.[21] Moreover a comprehensive *in silico* approach to permeability has been introduced.[22] The cyclic peptide (**2**) has passive permeability and oral bioavailability rivalling that of CsA itself. Likewise, both (**2**) and CsA have EPSA values indicating low polarity: 61.5 and 69.9, respectively (Table 3). One question of interest was the impact of cyclization on the properties of (**2**), so an acyclic analogue (**3**) was prepared (Fig. 2) replacing one peptide bond with an *N*-methyl amide and eliminating one methyl from the Abu-1 sidechain in order to retain an equivalent total carbon count and thus minimize changes in overall physicochemical profile, in particular molecular weight and log *D*.

Table 1 Properties of (**2**) and CsA

Compound	MW (Da)	log *D* (7.4)	RRCK P_{app} (AB) × 10^{-6} cm s^{-1}
2	1019.38	3.29	5.4
CsA	1202.63	4.97	5.6

Table 2 Rat pharmacokinetics of (**2**) and CsA

Compound	Route[a]	Dose (mg kg^{-1})	CL_p (mL min^{-1} kg^{-1})	$AUC_{0-\infty}$ (ng h mL^{-1})	Vd_{ss} (L kg^{-1})	$t_{1/2}$ (h)	C_{max} (ng mL^{-1})	Oral F (%)	F_a (%)
2	i.v.[b]	1 (n = 2)	9.8	1740	0.92	2.9			
	p.o.[b,c]	5 (n = 2)		1480			461	17	20
CsA	i.v.[b]	10	3.5	48 300	1.2	6.0			
	p.o.[b,c]	10		13 800			1440	29	30

[a] Animal care and *in vivo* procedures were conducted according to guideline of the Pfizer Animal Care and Use Committee. Pharmacokinetic studies were conducted in male Wistar-Han rats. [b] Compound (**2**) was administered as a homogeneous solution in 10% Cremophor EL : 10% EtOH : 80% saline solution for intravenous (i.v.) and oral (p.o.) studies. [c] Oral pharmacokinetics studies were conducted in fasted animals.

Table 3 Comparison of (2), (3) and CsA

Compound	MW (Da)	log *D* (7.4)	RRCK P_{app} (AB) × 10^{-6} cm s^{-1}	EPSA
2	1019.38	3.29	5.4	61
3	1020.73	3.43	0.6	66
CsA	1202.63	4.97	5.6	69

In silico modelling and NMR studies of CsA and analogues

Interestingly, (3) had an EPSA of 66 nearly matching that of CsA and indicative of its ability to hide polarity when in a hydrophobic environment. The cellular permeability of (3), on the other hand, was much less than (2), with RRCK P_{app} = 0.6 × 10^{-6} cm s^{-1}. Contemporary *in silico* methods of assessing permeability are more consistent with the EPSA values than the disparate RRCK values, indicating that these calculations are missing an important aspect of the physics in these complex systems. Physics-based modelling of (2), (3), and CsA (Table 4), using the method of Leung[20] gave very similar results for all three, both for membrane insertion and permeability overall and, in particular, appears to underestimate the permeability of the cyclic peptides (2) and CsA. Likewise, the predicted conformations indicate that (2) and (3) are equally able to adopt compact (R_{gyr} < 7 Å) low-polarity (3D-PSA < 100 $Å^2$) conformations with properties favourable for membrane permeation.

The disconnect in membrane permeability between (2) and (3), as measured by RRCK, therefore appears not to be attributable to differences in the permeating conformation itself. Rather, the greater intrinsic flexibility of the acyclic (3) may lead to reduced permeation either by impeding diffusion across the membrane or by adopting a far greater conformational space in the aqueous environment, leading to a large entropic loss upon "conformational focusing" to adopt conformations capable of traversing the membrane. Even sophisticated physics-based *in silico* computations of permeability may not adequately address these factors, as indicated by the similar predicted log (RRCK) for (2) and (3) (Table 4). The molecular descriptor of rotatable bond count and impact on pharmacokinetic profile is also increased in (3) and should be considered.[23]

NMR experiments in DMSO indicate that compound (3) has greater conformational flexibility than (2) in polar solvents, lending credence to the disparate "conformational focusing" hypothesis. The temperature coefficients for the cyclic peptide (2) are consistent with the presence of two intramolecular hydrogen bonds (IMHB's) at Val-4 and Ala-6 (Table 5). The temperature coefficients for the linear peptide (3) are consistent with no IMHB's using the cut-off of 4.0 ppb K^{-1}.[24] At best, (3) has two weak IMHB's for Ala-6 and D-Ala-7, indicative of a more flexible, less compact conformation (Table 5).

Modelling of the conformations of (2) identified conformations consistent with the IMHBs as well as seven moderate or strong NOE's identified in NMR experiments. The conformation with the lowest deviation from both expected IMHBs and NOE constraints is shown (Fig. 3). The IMHB pattern is significantly different from that seen in CsA, with Val-4 H-bonding to the carbonyl of neighbouring Sar-2 and Ala-6 forming a transannular H-bond to the carbonyl of MeLeu-3. This conformation also contains two *cis*-peptide bonds: between Abu-1 and Sar-2 and between Val-4 and MeLeu-5 which are supported by α–α NOE's for the adjacent residues.

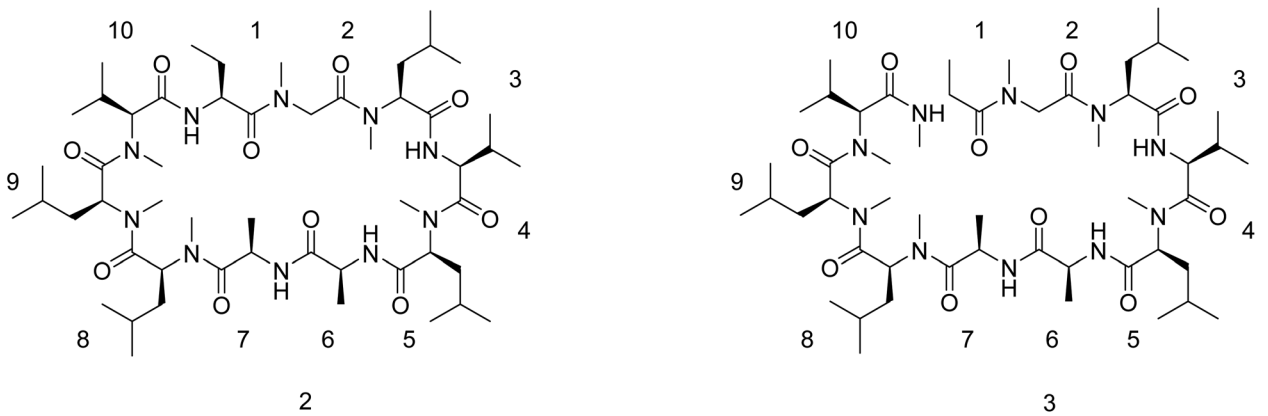


Fig. 2 Cyclic (2) and acyclic (3) analogues.

Table 4 Calculated properties of the predicted permeating conformations of (2), (3), and CsA

Compound	Predicted RRCK P_{app} (AB) × 10^{-6} cm s^{-1}	Δ*G* insert (kcal mol^{-1})	IMHB	TPSA ($Å^2$)	3D-PSA ($Å^2$)	R_{gyr} (Å)
2	0.7	7.3	2	238.3	77	6.1
3	0.8	6.3	2	238.3	94	5.7
CsA	0.3	7.2	2	278.8	119	6.5

Table 5 Temperature dependence and coupling constants of the NH protons in DMSO-d_6

2	δ (ppm) 295 K	$\Delta\delta/T$ (295–325 K) ppb K^{-1}	^3J HN-HCα 295 K (Hz)	3	δ (ppm) 295 K	$\Delta\delta/T$ (295–325 K) ppb K^{-1}	^3J HN-HCα 295 K (Hz)
Abu-1	8.28	10.0	9.7	Abu-1	7.82	6.2	NA[a]
Val-4	8.37	3.3	6.6	Val-4	7.99	9.1	8.3
Ala-6	8.32	2.6	5.8	Ala-6	8.04	4.6	7.5
D-Ala-7	7.91	8.8	7.7	D-Ala-7	7.54	4.2	7.5[b]

[a] Quartet. [b] Overlap with minor conformation.

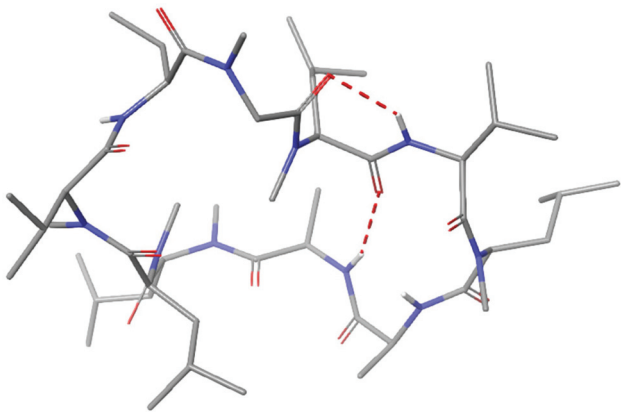


Fig. 3 Model of (2) in DMSO using constraints from NMR.

The 3D-PSA of this conformation is 124 Å^2, significantly higher than the predicted permeating conformation 77 Å^2. This indicates that despite the smaller ring size, the peptide may maintain enough flexibility to expose some polarity, though it appears significantly less dramatic than the structural inversion seen in CsA itself.[25] No equivalent conformation can be determined for (3) because NMR indicates it has only weak intramolecular hydrogen bonding, at best, and has relatively few NOESY correlations. It is likely to be a random coil in solution.[26]

A separate computational analysis of low-energy conformations of (2) and (3), using the Monte Carlo Multiple Minimum method with a DMSO dielectric (Small-Molecule Drug Discovery Suite 2016-4, Schrödinger, LLC, New York, NY, 2016) identified 418 conformations for (2) and 632 conformations for (3) within 5 kcal mol^{-1} of the respective low-energy conformations (Fig. 4). The analysis shows 10 cluster centroids of each from conformational clustering of these low energy conformations and indicates that while (3) may have some beta turn character, it appears overall less structured.

Conclusions

There have been many publications on the impact of cyclisation of peptides on oral bioavailability and there is general agreement that cyclisation reduces the proteolytic liabilities by preventing the peptide from assuming an open saw tooth or beta strand conformation that is required for many proteases to recognize and cleave substrates.[27] The impact of cyclisation on the membrane and cell permeability of peptides is a more open question with publications arguing that it assists while others suggest that the effects are negligible or due solely to improved proteolytic stability rather than permeability.[28] The *in vitro* and *in vivo* pharmacokinetic data of CsA analogue (2) demonstrates proof of principle that a library of cell permeable and orally bioavailable analogues can be prepared for broad screening utility. Furthermore the significantly reduced permeability of acyclic analogue (3) as measured in RRCK cells challenges our framework for understanding permeability of acyclic systems. Further studies are underway to improve our understanding of these important therapeutic molecules and potential for drug discovery.

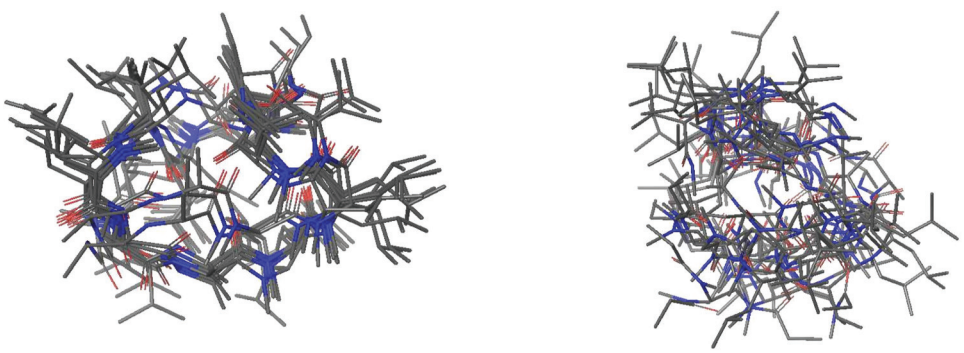


Fig. 4 Ten low-energy conformational clusters for (2) left and (3) right.

Acknowledgements

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