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NMR characterization and conformational analysis of a potent papain-family cathepsin L-like cysteine protease inhibitor with different behaviour in polar and apolar media

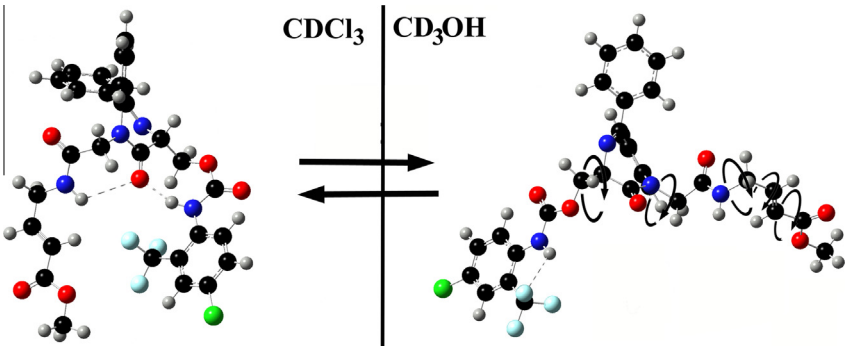
Archimede Rotondo [a,*], Roberta Ettari [b], Maria Zappalà [c], Carlo De Micheli [b], Enrico Rotondo [a]

[a] Dipartimento di Scienze Chimiche, University of Messina, Viale F. Stagno d'Alcontres 31, 98166 Messina, Italy
[b] Dipartimento di Scienze Farmaceutiche, University of Milan, Via Mangiagalli, 25, 20133 Milano, Italy
[c] Dipartimento di Scienze del Farmaco e dei Prodotti per la Salute, University of Messina, Viale Annunziata, 98168 Messina, Italy

HIGHLIGHTS

- We chose the ester as interesting case study because of its biological properties.
- The retention of the compound in apolar media focused our curiosity on this system.
- NMR data prove different structural features in methanol and chloroform.
- Intramolecular interactions play a key role in the detected conformational switches.
- Only the dynamic conformation typical of polar media can arise the biological activity.

GRAPHICAL ABSTRACT



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ABSTRACT

We recently reported the synthesis, of a potent papain-family cathepsin L-like cysteine protease inhibitor, as new lead compound for the development of new drugs that can be used as antiprotozoal agents. The investigation of its conformational profile is crucial for the in-depth understanding of its biological behaviour. Our careful NMR analysis has been based on the complete and total assignment of ^{1}H, ^{13}C, ^{15}N and ^{19}F signals of the molecule in both $CDCl_3$ and CD_3OH, which could reproduce in some way a scenario of polar and not polar phases into the biological environment. In this way it has been unveiled a different behaviour of the molecule in polar and apolar media. In $CDCl_3$ it is possible to define stable conformational arrangements on the basis of the detected through space contacts, whereas, in CD_3OH a greater conformational freedom is envisaged: (a) by the overlap of any of the CH_2 diastereotopic resonances (unable to distinguish asymmetric molecular sides because of the free rotation about the single bonded chains), (b) by the less definite measured vicinities not consistent with just one conformation and (c) by the evident loss or switching of key intramolecular hydrogen interactions.

Introduction

Neglected tropical diseases (NTDs) are a group of chronic disabling infections affecting over one billion people, two of the most relevant diseases are malaria and Human African Trypanosomiasis (HAT) [1]. Malaria is the most widespread and severe tropical disease, it is caused in humans by several species of the *Plasmodium* genus, being *P. falciparum* together with *P. vivax* responsible of more than 95% cases of malaria [2]. On the other hand, HAT is caused by protozoa of *Trypanosoma* genus: *T. brucei gambiense* and *T. b. rhodesiense* which are responsible for the transmission

* Corresponding author. Tel.: +39 090 6765714; fax: +39 090 393756.
E-mail address: arotondo@unime.it (A. Rotondo).

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of the disease to humans, causing chronic and acute forms of the pathology respectively [3]. The currently available therapeutic protocols are characterized by many drawbacks such as poor efficacy, toxicity, parasites resistance to drugs, cost and route of administration [4–6]. This situation urges to identify new targets for malaria and HAT treatment. With this aim we focused our attention on the cathepsin L-like cysteine proteases, falcipain-2 (FP-2) and rhodesain, which have been validated as promising targets for the treatment of protozoan infections [7,8].

In particular FP-2 of *P. falciparum*, takes part to the cleavage of the cytoskeletal elements of the erythrocyte membrane and it is involved in the haemoglobin degradation. On the other hand, rhodesain of *T. b. rhodesiense*, is responsible for the degradation of the blood–brain barrier (BBB) and for the immune evasion.

In this context, our research group has been actively involved in the synthesis and development of papain-family cysteine protease inhibitors [9,10]. Among all the synthesized compounds the most promising inhibitor was proven to be the α,β-unsaturated ester **1** (Scheme 1), characterized by the presence of a 1,4-benzodiazepine (BDZ) scaffold, introduced internally to a peptide backbone, in which the fused aromatic ring mimics a phenylalanine residue at the P2 site, strongly preferred in this position from both proteases, while the hydroxyl group of the scaffold has been employed to tie substituents able to establish additional interactions with the S3 site of the target enzymes. At the C-terminal moiety an α,β-unsaturated ester has been introduced as warhead, able to act as a Michael acceptor, thus leading to an irreversible alkylation of the enzyme targets.

The use of an appropriate scaffold, incorporated into a characteristic peptide sequence, has been selected because this strategy could be particularly advantageous in terms of potency and selectivity, as described also in our recent studies on other proteases [11].

The α,β-unsaturated ester **1** was proven to possess an extraordinary potency towards FP-2 (k_{2nd} = 3,571,000 M^{-1} min^{-1}) and a binding affinity (K_i) of 17 nM [12]. Compound **1** was screened also against rhodesain towards which it showed a moderate potency (k_{2nd} = 34,600 M^{-1} min^{-1}) and a K_i value of 0.81 μM. Overall an excellent selectivity has been detected towards the protozoan cysteine proteases with respect to the papain-family human cysteine proteases cathepsin B (k_{2nd} = 6000 M^{-1} min^{-1} and K_i = 7.3 μM) and L (K_i = 8.4 μM). Additionally, when tested on *P. falciparum*, ester **1** showed a good profile with an IC_{50} = 12 μM. However the discrepancy between the activity of **1** against the enzyme and the parasite (nanomolar *versus* micromolar) might be explained by the difficulty for this inhibitor to cross the parasite membranes. Consequently, we evaluated its apparent permeability (P_{app}) across the Caco-2 monolayer with an obtained medium permeability of **1** due to a low mass balance (sum of amount in the apical and basolateral chambers) which has been calculated to amount to 45%. This poor mass balance could be attributed to the strong interactions of 1 with the components of cell membranes, which are supposed to be strong because of the high lipophilicity of the molecule. In order to account for its behaviour in the physiological conditions, herein we report the NMR structural characterization and conformational analysis of **1** in both, polar and apolar media.

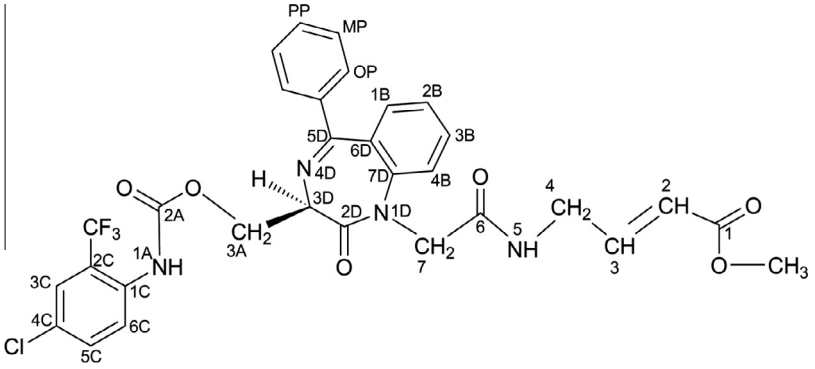
Experimental

Synthesis of 1

The synthesis of compound **1** has been previously reported by our group, [12] and it is briefly shown in Scheme 2. The *R* enantiomer of the Garner's ester **2**, was firstly converted into key intermediate **3** which, at reflux under acidic conditions, afforded the 3-hydroxymethyl substituted 1,4-benzodiazepine **4** through Boc deprotection of the nitrogen, cleavage of the oxazolidine ring and intramolecular condensation to form an imino bond. Intermediate **5**, obtained from compound **4** *via* a series of standard reactions (Scheme 2), was then condensed with commercially available allylamine to afford unsaturated amide **7**. Such a derivative was desilylated and the free primary alcohol reacted with 4-chloro-2-trifluoromethylphenyl isocyanate to afford carbamate **7**, a fully functionalized scaffold at the P1–P3 region. The terminal olefin was used to insert the required "warhead" *via* the cross metathesis methodology, using the Hoveyda–Grubbs 2nd generation catalyst (a phosphine-free *N*-heterocyclic carbene ruthenium complex), which actually represents the catalyst of choice for these kind of reactions [13].

Analytical methods

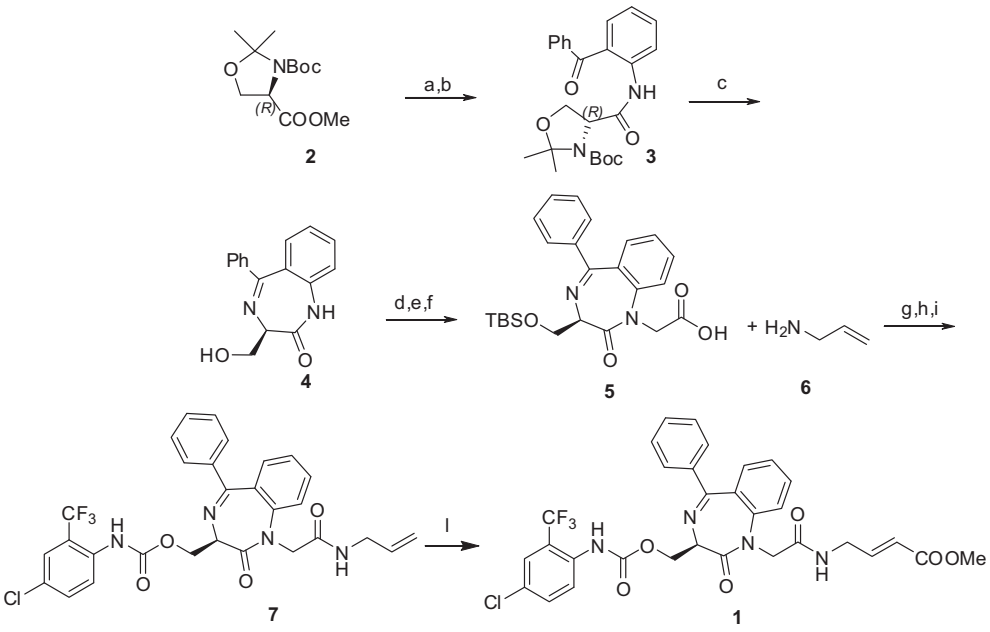
^1H, $^{13}C\{^1H\}$ and $^{15}N\{^1H\}$ NMR spectra of **1** were recorded on Bruker Avance 300 MHz NMR spectrometer equipped with a BBI probe and operating at frequencies of 300.13, 75.47, 30.42 MHz; many experiments were double checked on a Varian 500 MHz spectrometer equipped with a ONE_NMR probe and operating at 499.74, 125.73, 50.65 MHz respectively; moreover it was necessary to record ^{19}F spectra at 469.65 MHz. Compound **1** was dissolved in 500 μL of $CDCl_3$ (10 mg) and in 500 μL of CD_3OH (saturated solution, about 3 mg). Calibration was attained using as internal



1

(*R*,*E*)-methyl 4-(2-(3-((4-chloro-2-(trifluoromethyl)phenylcarbamoyloxy)methyl)-2-oxo-5-phenyl-2,3-dihydro-1*H*-benzo[*e*][1,4]diazepin-1-yl)acetamido)but-2-enoate

Scheme 1. Molecular representation and atom labelling scheme for **1**.



Scheme 2. Reagents and conditions: (a) LiOH, MeOH, 0 °C-rt, 6 h; (b) *i*-BuOCOCl, NMM, CH_2Cl_2, 0 °C-rt, 30 min, then 2-aminobenzophenone, reflux, 20 min, rt, 12 h; (c) HCl/MeOH, reflux, 5 h, then $NaHCO_3$, MeOH, rt, 12 h; (d) TBS-Cl, imidazole, CH_2Cl_2, 0 °C-rt, 12 h; (e) NaH, 0 °C, 1 h then $BrCH_2COOEt$, rt, 12 h; (f) LiOH, MeOH, 0 °C-rt, 6 h; (g) HATU, CH_2Cl_2, rt, 12 h; (h) TBAF, THF, rt, 5 h; (i) *4-Chloro-2-(trifluoromethyl)*phenyl *isocyanate*, rt, 12 h; (l) methyl acrylate, Hoveyda–Grubbs II generation catalyst, CH_2Cl_2, 100 °C, 2 h, MW.

standard residual proton signal of the solvent ([D_3] methanol: δ = 3.31 ppm; $CDCl_3$ δ = 7.26 ppm) and the ^{13}C solvent septuplet δ = 49.0 ppm and triplet δ = 79.5 respectively [14]. ^{15}N calibration was referred to the CH_3NO_2 as external standard (90% CH_3NO_2 in CD_3OH δ = 380.5 ppm).

Complete and unambiguous assignment was pursued by several 1D and 2D homo- and hetero-nuclear NMR experiments such as 2D-TOCSY, 2D-NOESY, ^{13}C-HSQC, ^{13}C-HMBC and ^{15}N-HSQC. Table 1 reports the assigned resonances in both solvents; this is at the basis of any further consideration. According to the many data available we can assess that the potent cysteine protease inhibitor is more soluble in apolar solvents such as chloroform. In the raw solvent though, it is not possible to study the conformational features because the residual DCl molecules involve amidic protons (especially 5-N*H*) in some kind of dynamic prototropic equilibrium. This is the reason why, after many assays, we decided to add small amounts of insoluble KOH to the sample in order to get rid of the unwanted line-broadening effects (because of the slight shifts caused by the base, H_2O would be the best choice but it does not separate from the chloroform phase giving a very nasty signals around 4.8 ppm). On the KOH saturated sample we performed the exact measurement of the NMR constants with a complete and total assignment of the ^1H and H-bound ^{13}C resonances. It was also possible to distinguish the different geminal protons on the basis of NOE dipolar couplings (Fig. 1, Tables 1 and 2 and supplementary data).

As an alternative solvent we have chosen the non-conventional CD_3OH for the following reasons: (a) because of its capability to dissolve enough of the molecule to record ^1H, ^{13}C and ^{15}N chemical shifts (*cs*); and (b) to detect also the N—H amidic resonances in a polar protic solvent somewhat reminding a biological environment (Fig. 1, Tables 1 and 2 and supplementary data).

Selective DPFGSE 1D NOE [15] spectra, which were cleaner and more sensitive, have been integrated by the Bruker Topspin software package (version 2.5) and Mestrenova (version 6.0.2-5475). These integration data were then converted into proton-proton average distances using the two-spin approximation and the geminal 3A-CH_2 NOE integration as reference (1.78 Å). The average distances extracted by many experiments at different mixing times [16] are then evaluated and reported for both studied environments (Table 2).

Theoretical models

The model of inhibitor **1** was built in the MOLEFACTURE routine of the VMD program operating with AMBERTOOLS PM3 minimization methods. Several models were treated by GAUSSIAN03 software package [17] with semi-empirical methods at the MP3MM level [18]. Some of the meaningful models are herein reported to better understand the NMR findings.

Results and discussion

Preliminary remarks

Conformational analysis of BDZ derivatives have attracted the attention of many researchers [19] because of the known pharmacological activities of these constructs which are related to their structure according to the known "structure–activity relationship" (SAR) [20]. The seven-membered ring might theoretically assume many conformations [21], but, because of specific substituents [20] or coordination modes [22], only few of the conformers keep a thermodynamic relevance. This is demonstrated by both theoretical [23] and experimental evidences [24]. The molecule **1** can assume just two BDZ boat conformations with the benzo-fused ring upward (facing the 3A-CH_2) or downward (facing the 3D-CH). We think these conformations are both populated in solution, but NOE confirms always the prevalent presence of the latter. This was also the biologically active conformation [10c,10d]. Our challenge is to evaluate the conformational behaviour of the whole **1** molecular scaffold in different solvents since it may shed light on the biological behaviour of this inhibitor.

Spectroscopical discussion

Conformational analyses of biologically interesting compounds, by itself, is a fundamental step towards the whole knowledge of

Table 1
^{1}H *cs* of compound **1** in chloroform and in methanol at 298 K.

Code	^{1}H $CDCl_3$	^{1}H CD_3OH	^{1}H & ^{19}F Delta	Parent ^{13}C $CDCl_3$	Parent ^{13}C CD_3OH	^{13}C & ^{15}N delta
CH_3	3.69	3.70	+0.01	51.9	52.0	+0.2
2-CH	5.84	5.97	+0.13	121.7	121.7	0.0
3-CH	6.82	6.90	+0.08	143.6	145.9	+2.3
4-CH2	4.00	4.01	+0.01	40.4	41.2	+0.8
5-NH	6.59	8.62	**+2.03**	109.9[b]	110.3[b]	+0.4
7-CH_2	4.31	4.61	+0.3	53.0	51.9	−1.1
7′-CH_2	4.67		−0.06			
4B-CH	7.74	7.58	−0.14	123.0	123.4	+0.4
3B-CH	7.62	7.66	+0.04	133.0	133.4	+0.4
2B-CH	7.28	7.31	+0.03	125.5	126.0	+0.5
1B-CH	7.36	7.28	−0.08	131.0	131.5	+0.5
OP-CH	7.61	7.58	−0.02	130.2	130.9	+0.7
MP-CH	7.39	7.43	+0.04	128.6	129.2	+0.8
PP-CH	7.47	7.51	+0.04	131.6	131.9	+0.3
3D-CH	4.05	4.108	+0.05	61.9	63.0	+1.1
2A-CH_2	4.94	4.85	−0.09	65.3	66.2	+0.9
2A′-CH_2	5.06		−0.21			
1A-NH	7.05	8.90	**+1.85**	95.7[b]	93.8[b]	−1.9
3C-CH	7.55	7.67	+0.12	126.3	127.1	+0.8
5C-CH	7.49	7.62	+0.13	133.0	133.9	+0.9
6C-CH	8.06	7.70	**−0.37**	124.7	130.8	**+6.1**
CF3	−65[a]	−62.70	+3.3			
		−62.71				

In bold character there are the most relevant cs variations.
[a] Concerns ^{19}F resonances instead of ^{1}H.
[b] Refers to ^{15}N resonances instead of ^{13}C.

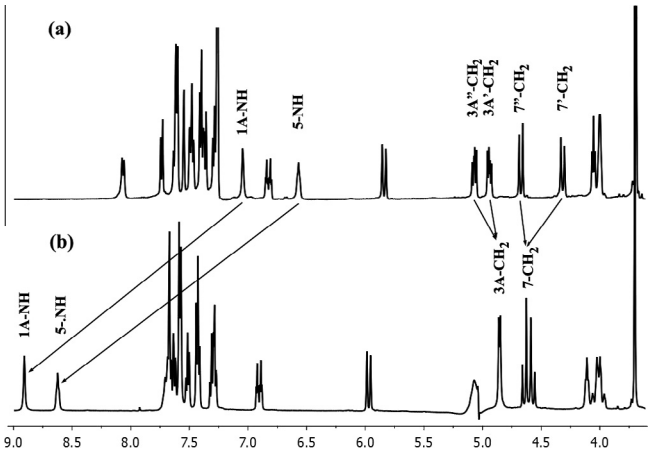


Fig. 1. ^{1}H NMR spectra at 500 MHz: (a) in KOH saturated $CDCl_3$ at 25 °C; (b) in CD_3OH at 5 °C with the O—H suppression. The complete ^{1}H assignment is possible showing the crucial differences.

the interaction mechanisms occurring in the chemistry of life [25]. Moreover, many biological molecules travelling within biological systems pass through many different environments; this change of media often triggers conformational mutations. Being the title compound soluble in chloroform, it was straightforward to perform the first NMR analysis in this solvent. From the ^{1}H NMR spectrum it was immediately clear that two over three of the CH_2 methylene signals look split according to diasterotopic behaviour of these nuclei. Usually this effect is remarkable in the case of rather rigid conformations; consistently, after the complete and total assignment of the ^{1}H and ^{13}C signals, it was possible to observe different dipolar couplings ("through the space" NOE information) for the mentioned geminal nuclei. The many defined 1D NOE data allowed the evaluation of the average distances (Table 2): provided there is a certain conformational freedom, it is possible to find a PM3MM minimised conformation which suits the main detected contacts and some other experimental data (Fig. 2). First we would like to point out the long range contact

Table 2
^{1}H average distances detected by 1d NOE integrations at 298 K.

Contact in $CDCl_3$		$CDCl_3$ (Å)	Contact in CD_3OH		CD_3OH (Å)
2-CH	CH3	4.85	2-CH	CH3	5.21
2-CH	5-NH	3.20	2-CH	5-NH	3.55
2-CH	4-CH_2	3.71	2-CH	4-CH_2	4.18
3-CH	5-NH	4.85	3-CH	5-NH	4.70
3-4CH	CH3	n.d	3-4CH	CH3	5.38
3-CH	4-CH_2	2.94	3-CH	4-CH_2	3.45
4-CH_2	5-NH	2.94	4-CH_2	5-NH	3.05
5-NH	7′-CH_2	3.60	5-NH	7-CH_2	2.98
5-NH	7″-CH_2	2.54			
5-NH	4B-CH	3.95	5-NH	4B-CH	4.47
5-NH	3B-CH	4.04	5-NH	3B-CH	nd
7′-CH_2	**7″-CH_2**	**1.78**	–	–	–
7″-CH_2	4B-CH	2.62	7-CH_2	4B-CH	2.78
7′-CH_2	4B-CH	2.54			
7′-CH_2	3B-CH	4.32	7-CH_2	3B-CH	nd
7″-CH_2	3B-CH	4.04			
3D-CH	1A-NH	4.32	3D-CH	1A-NH	nd
3D-CH	4B-CH	4.04	3D-CH	4B-CH	3.98
3D-CH	3A′-CH_2	2.51	3D-CH	3A-CH_2	2.66
3D-CH	3A″-CH_2	2.62			
3A′-CH_2	3A″-CH_2	1.76	–	–	–
3A′-CH_2	1A-NH	3.85	3A-CH_2	1A-NH	nd
3A′-CH_2	OP-CH	4.32	3A-CH_2	OP-CH	4.23
3A″-CH_2	OP-CH	4.85			
3A″-CH_2	6C-CH	4.32	3A-CH_2	6C-CH	nd
2B-CH	3B-CH	nd	**2B-CH**	**3B-CH**	**2.50**
1B-CH	OP-CH	nd	1B-CH	OP-CH	3.0

Entries in bold were used as reference to determine all the other average distances.

3D-CH/4BCH & 3D-CH/1BCH assessing the boat conformation of the BDZ with the benzo-fused ring on the same side of the 3D-CH group; on the other side the close contact 5-NH/2-CH witnesses that there is a certain stiffness of the warhead conformation. BDZ conformation, retained also in CD_3OH (Table 2), actually does not diverge too much from the supposed active conformation inside the macromolecular model of the complex [10c], however the warhead and the carbamoyl pendant chain look quite over folded in $CDCl_3$.

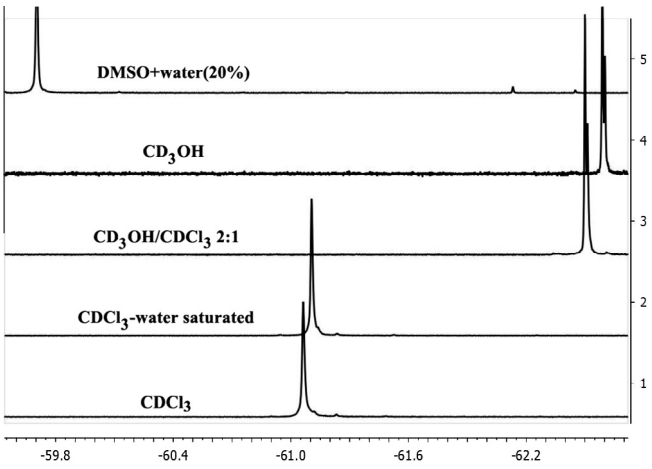


Fig. 2. ^{19}F Spectrum in progressively more polar environments: according to our knowledge [26] the 1A-NH···CF3 interaction gets more and more permanent in pure CD_3OH eventually fading out in strong polar environments.

In CD_3OH, the most remarkable NMR spectral changes concern the CH_2 signals which are not any more split. This is *per se* the evidence that dihedral angles around CH_2 groups enhanced their degrees of freedom. Specifically, by adding just few microliters of CD_3OH in the $CDCl_3$ solution, 3A-CH_2 signals quickly coalesce into one resonance whereas the other geminal 7-CH_2 protons begin to get closer keeping though their own identity. This experiment reveals that dihedral angles around 3A-CH_2, which are in some way constrained in chloroform, seem right away to free in polar-protic solvents.

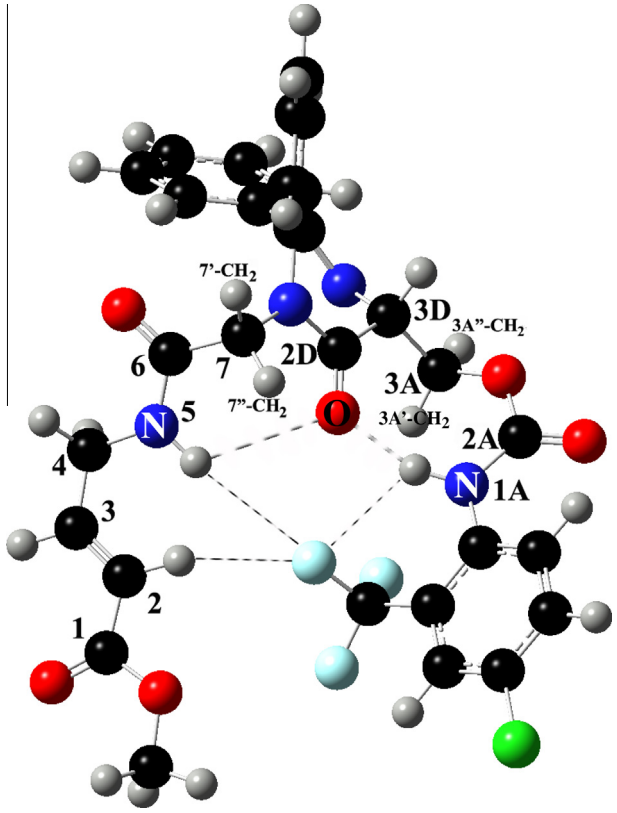


Fig. 3. Sound conformation in chloroform as minimized with semi-empirical methods PM3MM: most of the NOE contacts are consistent with this conformation and the entangled intramolecular interactions match the other NMR evidences. This is the most populated conformation in chloroform but it is not the only one.

The ^{19}F spectrum, showed two different signals with about 1:2 integration ratio in CD_3OH, shielded respect to the single resonance observed in $CDCl_3$; on another hand, in strong dipolar and protic solvents chemical shift is well deshielded (Fig. 2). This suggest CF_3 group is deep inside an intramolecular network of dipolar interactions in $CDCl_3$ whereas, in CD_3OH a permanent intramolecular 1A-NH···CF_3 hydrogen bond has to be invoked; moreover this is known to play a very important role in respect to the biological activity of many substrates [26], including the present one [10c]; in strong polar media this interaction is demonstrated to fade out because of the competing solvent interactions (Fig. 2 and supplementary data).

The permanent 1A-NH···CF_3 interaction in CD_3OH is confirmed by the lack of any 1A-NH through the space contacts despite the $CDCl_3$ detection of few long-range couplings. According to our knowledge it would be pretty unusual that intra-molecular H-bonds are favoured in polar solvents, however, the overall data together with few minimizations run by semi-empirical methods at PM3MM level, revealed the competing and/or alternative presence of the 1A-NH···O=C intra-molecular interaction which is reasonably prevailing in chloroform (reduction of the dipolar moment) accounting also for the evident rigid conformation around the 3A-CH_2 torsional angles (Fig. 3) which easily gains flexibility in presence of possible H-donors (protic solvents).

Generally speaking, the folded conformation presents a crowded network of loose intramolecular dipolar interactions

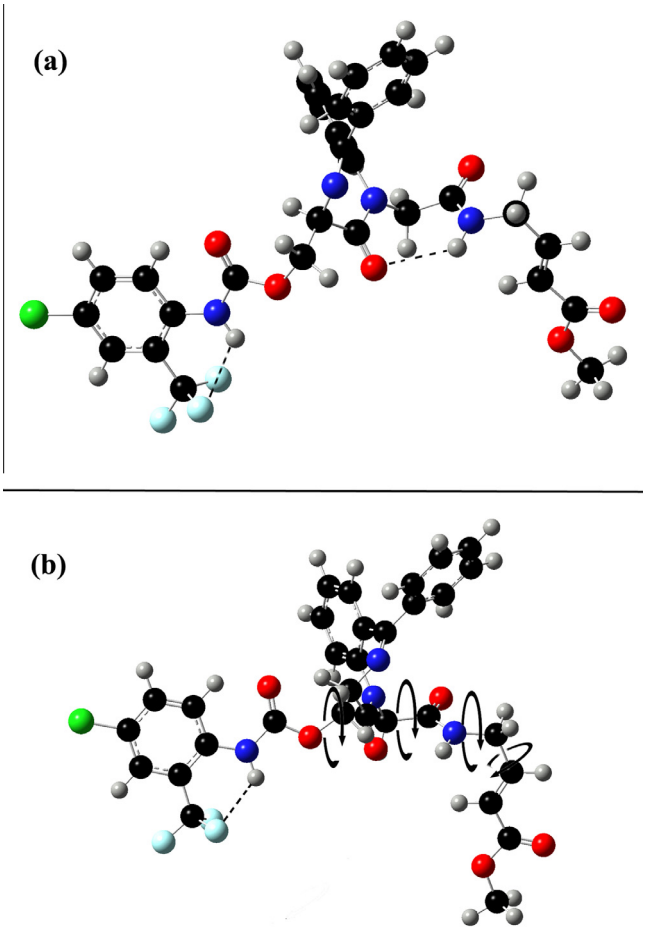


Fig. 4. (a) Representation of a stable conformation (PM3MM global minimum) still presenting one of the intramolecular interactions involving the endocyclic carbonyl group and (b) free conformation detected in pure CD_3OH, the most dihedral angles show free rotation.

perfectly matching the main detected $CDCl_3$ NOE. It is easy to understand that the structure opens up when many possible solvent-dipolar interactions occur (Fig. 4). Interestingly, beyond the amide protons, the most relevant ^1H chemical shift differences concern 6C-C*H* and 4B-C*H* (Table 1) and the most shifted ^{13}C is the 6C-*C*H, these changes well match the intramolecular 3A-NH···3D-C═O switching. The presence of 3A-NH intramolecular hydrogen interactions in both solvents is also confirmed by the ^{15}N NMR resonance of 1A-*N*H which is at much lower frequencies from what expected; it is more than 15 ppm downshift respect to the 5-*N*H which should be theoretically more shielded (Table 1). Also the comparison of many ^1H NMR profiles and line-broadening measurements highlights 1A-N*H* resonance is always the less involved in chemical proton exchange testifying this site is pretty protected by the molecule itself (this is not the case of strongly polar solvents, see supplementary material and Fig. 2). Also the other amidic 5-N*H*, in $CDCl_3$, is involved in a weaker intramolecular H-bond with the endocyclic carbonyl group (3D-C═O), this is keeping the 7-CH_2 resonance splitting.

In summary we can talk about a whole intramolecular bonding network which is gradually switching from a folded structure, overwhelming in $CDCl_3$ (Fig. 3), to a much more dynamically opened structure in pure CD_3OH where solvent-intermolecular interactions prevail but the local 1A-NH···CF_3 turns out favoured (Fig. 4). Experiments with the mixture of solvents show that, once the aromatic branch gains freedom, dihedral angles on the aliphatic warhead branch are still a little locked as also witnessed by the global minimum found by theoretical calculations (Fig. 4a). Another important difference in the two solvents has to be underlined is related to the warhead fragment: in MeOH, the simultaneous presence of the couples 5-NH/2-CH & 5NH/3-CH; 4-CH_2/2-CH & 4-CH_2/3-CH; CH_3/2-CH & CH_3/3-CH dipolar couplings, accounts for a certain flexibility of the terminal warhead in respect to the more embedded $CDCl_3$ arrangement (Fig. 4b).

Conclusions

We carried out a thorough conformational analysis of the inhibitor 1 in apolar and polar media, assuming that these can simulate specific biological environments. Although the presented ester is less soluble in polar and protic solvents, these media are necessary for its biological activity; indeed, whereas the aryl carbamoyl moiety has to be locked by the 1A-NH···CF_3 interaction to functionally occupy its specific S3 pocket [10c], the fluxional features of the remaining fragments are necessary to seek for the suitable conformation yielding crucial interactions within the binding site, thus enabling the interaction with the catalytic triad Cys/His/Asn.

We have found that this compound is stable in apolar media such as chloroform (and the lipophilic membranes) by a reduced polarity brought about by a network of several intramolecular interactions involving polar residues (NH and carbonyl groups but also the CF_3 and polar C—H residues). The folded conformation, mainly retained in apolar media, is not suitable to fit the catalytic sites of the protease under investigation. Intermediate conditions, generated by mixtures of solvents, also revealed that the carbamoyl chain is the first one gaining freedom by the cleavage of the 1A-NH—2D-C═O interaction, while in a polar and protic environment (pure CD_3OH) also the weaker interaction 5-NH—2D-C═O fades out leaving room to interactions with the solvent. These last ones appear to be of utmost importance to impart to the unsaturated ester a conformational freedom necessary to induce a productive fit with the catalytic sites which produces the observed biological activity. To complete this analysis we have to report that, in very strong polar conditions (in d_6-DMSO and 20% of water), even the last amphiphilic 1A-NH—CF_3 interaction eventually fades out leaving room to many possible solvent-intermolecular interactions.

Acknowledgements

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molstruc.2014.07.046. These data include MOL files and InChiKeys of the most important compounds described in this article.

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