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Dipeptidyl α-fluorovinyl Michael acceptors: Synthesis and activity against cysteine proteases

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Abstract—The synthesis of novel dipeptidyl α-fluorovinyl sulfones using a Horner–Wadsworth–Emmons approach on *N*-Boc-L-phenylalaninal is described. Inhibitory assays against a *Leishmania mexicana* cysteine protease (CPB2.8ΔCTE) revealed low biological activity. Relative rates of Michael additions of 2′-(phenethyl)thiol with vinyl sulfone and α-fluorovinyl sulfone were determined, and ab initio calculations on several Michael acceptor model structures were performed; both were in agreement with the biological testing results.
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Cysteine proteases are peptide bond-cleaving enzymes. They make use of a nucleophilic thiol group that attacks the peptide carbonyl group, giving rise to the free amine and a peptidyl thioester—which after hydrolysis affords the carboxylic acid part. They have been divided into several clans, which are subdivided into several families (http://merops.sanger.ac.uk/). Cysteine proteases are very widespread in nature and hold cornerstone positions in the metabolism of both eukaryotic and prokaryotic organisms. Several inhibitors are involved in drug development programmes targeting human proteases for diseases such as osteoporosis, arterial thrombosis, rheumatoid arthritis, tumour invasion and metastasis and Alzheimer's disease.[1,2] Cysteine proteases are also considered as vital for protozoa such as *Plasmodium*, *Trypanosome* and *Leishmania* and inhibitors of falcipain and cruzain, two major parasitic cysteine proteases, are under investigation as possible treatment of malaria and Chagas' disease.[3]

Most of these inhibitors are derived from the corresponding peptide substrate of the target enzyme. They are modified at the P1 position where the amide group is substituted for a so-called warhead: an electrophilic group with high affinity for the active site thiol group. Frequently used warheads include ketones and nitriles affording reversible tight binding inhibitors or epoxides and Michael acceptors capable of reacting covalently with the thiolate affording irreversible inhibitors.[1]

One of the most interesting Michael acceptor groups are peptide vinyl sulfones and their analogues such as vinyl sulfonamides and vinyl sulfonate esters.[4] Vinyl sulfones selectively inhibit several cysteine proteases in a low nM range. Compounds such as **1** inhibit falcipains and cruzain and are important tools in antiprotozoal drug design and development (Fig. 1).[5–7]

Very few Michael acceptor inhibitors with substitutions on the α-vinyl carbon have been reported. An α-acetoxymethyl group (Fig. 2) affords compounds showing activity in an in vitro malaria model (IC_{50} = 10 nM). A double addition–elimination mechanism of action is claimed but detailed kinetic analysis of enzyme inhibition to verify this claim is not reported.[8]

Keywords: Cysteine proteases; α-Fluorovinyl Michael acceptors; Inhibitors.
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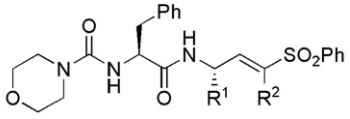


1, $R^1 = CH_2CH_2Ph$, $R^2 = H$ **2**, $R^1 = CH_2Ph$, $R^2 = H$ **3**, $R^1 = CH_2Ph$, $R^2 = F$

Figure 1. Fluorinated and non-fluorinated vinyl sulfones cysteine protease inhibitors.

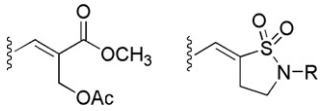


Figure 2. Different substitutions on the α-carbon of Michael acceptors.

Vinyl sultams (Fig. 2) as conformationally restricted analogues of vinyl sulfonamides were not active as inhibitors of papain and demonstrated weak activity against recombinant falcipain-2.[9] Our interest in fluoro-olefins[10] prompted us to evaluate the influence of an α-fluorine in a Michael acceptor type cysteine protease inhibitor. In the thermodynamically favoured conjugate addition, the proposed mechanism involves the rate-limiting reaction of a Michael acceptor olefin with a rapidly formed complex between thiol and tertiary base.[11] The reactivity of the addition might indeed be enhanced if the electrophilicity of the β-carbon of the Michael acceptor is increased. The introduction of an inductively electron withdrawing group on the α-carbon such as fluorine will increase the electron deficiency at C_α, but no prognosis can be made on how strong will be the effect on C_β. On the other hand, the lone pairs of fluorine might participate and show overlap with the existing conjugate π-system, in this way contributing as a mesomeric donor.

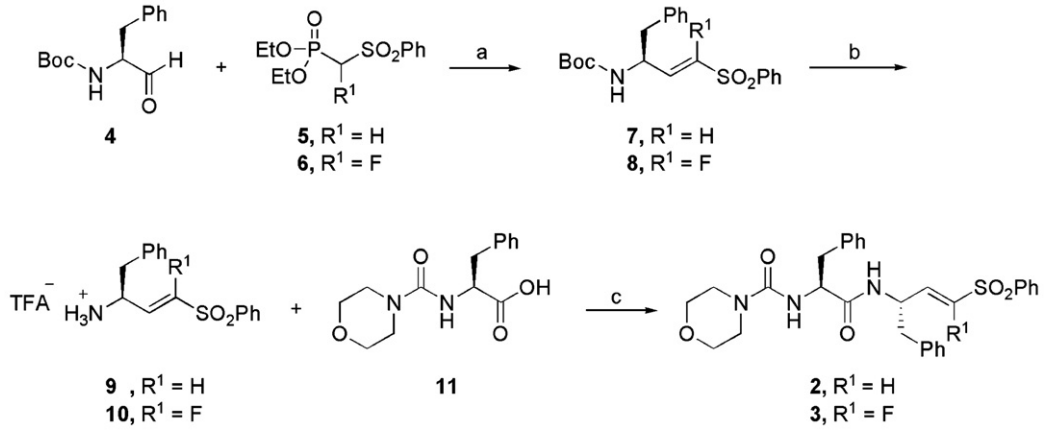
We used the vinyl phenylsulfone acceptor system and prepared reference compound **2** and its α-fluoro derivative **3**. We found compound **2** to be a potent inhibitor of the *Leishmania* cysteine protease CPB.[12]

Inhibitor **2** was synthesized (Scheme 1) using a Horner–Wadsworth–Emmons (HWE) reaction. Oxidation of the commercially available diethyl [(phenylthio)-methyl]phosphonate to the corresponding sulfone **5** afforded the HWE reagent. Reaction with *N-tert*-butoxycarbonyl-phenylalaninal[13] **4** yielded vinyl sulfone intermediate **7**.[14,15] It has to be noted that only the *E*-isomer of **7** was isolated (yield 52%) as revealed by ^{1}H NMR analysis (^3J_H = 15.2 Hz). After coupling the deprotected vinyl sulfone **9** to carboxylic acid **11**,[16] reference target compound **2** was obtained in an overall yield of 48%. The fluorophenyl vinyl sulfone **3** was prepared (Scheme 1) by a WHE reaction of *N-tert*-butoxycarbonyl-phenylalaninal **4** with the anion of diethyl 1-fluoro-1-(phenylsulfonyl)methanephosphonate **6**. The latter was generated from fluoromethyl phenyl sulfone[17] and diethyl chlorophosphate using LiHMDS. This resulted in both (*E*)- and (*Z*)-isomers **8.1** and **8.2**, which were separated on a small scale. Olefin geometries were assigned using coupling constant ($^3J_{HF}$ = 32 Hz and 20.2 Hz) and the isomeric ratio was found to be approximately 1:1. Racemisation was minimized by using reaction times of 2 h and isolating the diethyl 1-fluoro-1-(phenylsulfonyl)methanephosphonate **6** instead of the literature-described in situ generation, thus avoiding the use of two equivalents of strong base in the reaction mixture.[18] Coupling of carboxylic acid **11** with the deprotected fluorovinyl sulfone **10** provided the (*E*)- and (*Z*)-isomers **3.1** and **3.2** of our target compound.

We used three approaches in order to obtain information on the influence of α-fluorine substitution in a Michael addition substrate cysteine protease inhibitor: (i) an in vitro chemical approach with a simplified amino acid-derived fluoro-olefin **8**; (ii) a theoretical approach calculating the atomic charge distribution; and (iii) an enzymatic evaluation of the peptide-derived fluoro-olefin inhibitor **3** as a CPB inhibitor.

Inspired by the work of Reddick et al.,[15] relative rates of Michael additions of 2′-(phenethyl)thiol with vinyl sulfone **7** and α-fluorovinyl sulfones **8** were determined (Scheme 2).

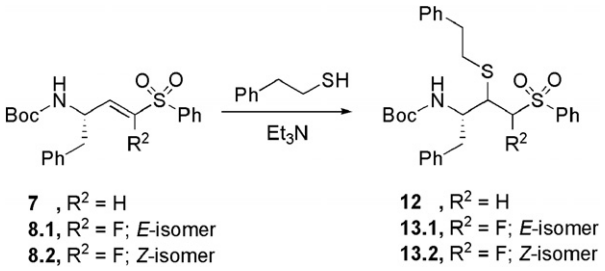
The reactivity of these Michael acceptors towards nucleophiles such as thiolates, serving as a model for the



4 **5**, R^1 = H **6**, R^1 = F **7**, R^1 = H **8**, R^1 = F

9 , R^1 = H **10**, R^1 = F **11** **2**, R^1 = H **3**, R^1 = F

Scheme 1. Synthesis of vinyl phenylsulfone **2** and phenyl α-fluorovinyl phenylsulfone **3**. Reagents and conditions: (a) LiHMDS, THF, −78 °C (if R^2 = F) or NaH, THF, 0 °C (if R^2 = H); (b) TFA, CH_2Cl_2 (1:1); (c) TBTU, NEt_3, DMF.



Scheme 2. Conjugate addition of thiolates to vinyl phenylsulfones.

active site cysteine residue, allows the evaluation of the electronic modifications due to the presence of a fluorine atom. Compared to the unsubstituted olefin geometry, the steric differences due to the presence of the fluorine atom will be limited because of its very small van der Waals radius (1.35 Å).

Relative reaction rates of base-promoted Michael additions of 2′-(phenethyl)thiol to acceptors **7** and **8** were measured using ^{1}H NMR spectroscopy. The integration of the vinylic proton signal indicates the presence of starting product, as a function of time several ^{1}H NMR spectra were taken in order to see the amount of vinyl sulfone consumed.

Fluorovinyl sulfone compounds **8.1** and **8.2** underwent no reaction in the given time. The vinylic protons of **8** ($\delta_{(E)}$ = 6.1 ppm and $\delta_{(Z)}$ = 5.8 ppm) remained unchanged during 1 h at room temperature. No difference in reactivity was observed in the (*E*)- and (*Z*)-isomers. The reference vinyl sulfone **7** however reacted to completion within 45 min; the identity of the addition product was confirmed by MS. The addition reactions were repeated afterwards and followed by TLC, starting product fluorovinyl sulfone **8** could still be detected after 48 h.

In conclusion, a lower reactivity of the Michael acceptor moiety towards thiolate nucleophiles is observed when an α-fluorine is introduced. The problem urges a theoretical chemistry approach using ab initio calculations to obtain charge densities on different atoms.

The effect of the α-fluorination of Michael substrates was studied by ab initio methods. As model systems, four different Michael acceptor groups were chosen: vinyl aldehyde, vinyl acid, vinyl ester and vinyl sulfone and their α-fluorinated derivatives (Fig. 3). The structures were optimized using the Gaussian03 program[19] at the B3LYP/6-311++G** level of theory. Subsequently, the atomic charges of the atoms in the molecules were calculated using the Hirshfeld method,[20] as implemented in the program STOCK.[21] The atomic charges of the backbones of the different molecules are presented in Table 1.

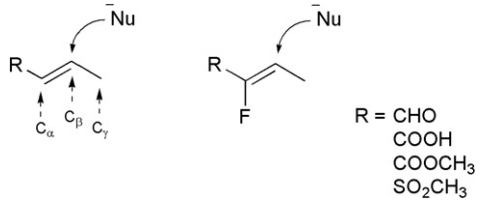


Figure 3. Michael acceptor model systems.

A general trend in the atomic charge distribution of the backbone is present. In the non-fluorinated compounds, an alternating pattern of the atomic charges is established, indicating the presence of a mesomeric system in those α, β-unsaturated molecules. The carbonyl carbon in the aldehyde, acid and ester molecules, as well as the sulfur atom in the sulfone molecule, are positively charged, causing a further polarization of the double bond between the α- and the β-carbon. This mesomeric effect creates a slightly positive charge on the β-carbon, making it suitable for a nucleophilic attack. Although the values for the charges on the β-carbon, presented in Table 1, may seem rather small (0.01 a.u.), one must consider the fact that the calculations are performed in the gas phase, as solvation effects are difficult to take explicitly into account. The effect of the mesomeric system is expected to be larger in solution and a basic environment, where the charge separation is further stabilized by the solute molecules and the mesomeric effect is strengthened by the presence of ionic molecules (e.g. acid and base).

Introducing a fluorine atom at the α-carbon results in a surprising effect: the positive charge at the α-carbon implies that the fluorine atom influences the charge density by means of a field effect, rather than a mesomeric donating effect. However, this field effect does not extend further to the β-carbon. The bond between the α-carbon and the β-carbon is further polarized, creating a partial negative charge on the latter. As a result, the β-carbon is no longer a suitable place for a nucleophilic attack. This additional negative charge on the β-carbon does not have an effect on the charge of the γ-carbon. Instead, the hydrogen atom on the β-carbon becomes more positive.

The negative charge on the β-carbon could also be explained by a mesomeric effect.[22] In one of the possible mesomeric forms, a double bond is formed between the

Table 1. The atomic charges (in a.u.) of the backbones of the studied compounds

αH		αF		αH		αF	
Aldehyde				Acid			
C(O)	0.11	C(O)	0.11	C(O)	**0.19**	C(O)	**0.19**
C_α	0.05	C_α	0.06	C_α	−0.06	C_α	0.06
C_β	0.01	C_β	−0.02	C_β	0.01	C_β	−0.02
C_γ	−0.07	C_γ	−0.07	C_γ	−0.07	C_γ	−0.07
Ester				Sulfone			
C(O)	0.18	C(O)	0.18	C(O)	0.47	C(O)	0.47
C_α	−0.07	C_α	0.06	C_α	−0.07	C_α	0.04
C_β	0.01	C_β	−0.02	C_β	0.01	C_β	−0.02
C_γ	−0.07	C_γ	−0.07	C_γ	−0.07	C_γ	−0.07

Table 2. Inhibition of *L. mexicana* cysteine protease CPB2.8ΔCTE with non-fluorinated (**2**) and fluorinated (**3**) Michael acceptor inhibitors

Compound	% Inhibition at 20 μg/mL	% Inhibition at 2 μg/mL
2	97 ± 1.9	71.3 ± 22.0
3.1	15.1 ± 15.9	0
3.2	38.7 ± 22.7	1.1 ± 1.9

*Mean ± standard deviation from three reactions.

fluorine and the α-carbon, resulting in a negative charge on the β-carbon. However, when bond lengths between the α- and β-carbons are examined, no significant changes are observed on the substitution of a hydrogen atom by a fluorine atom, the changes being smaller than 0.01 a.u., whereas a mesomeric effect would lead to the lengthening of this bond. Also, the average bond length between the fluorine atom and the α-carbon in the examined compounds is 1.35 a.u., which is a typical bond length for a single carbon–fluorine bond.

The presence of a mesomeric effect can be further excluded by examining the overall change in the charge density of the backbone as a result of the introduction of a fluorine atom at the α-carbon.[23] The total charge of the backbone becomes more positive by an average of 0.08 a.u., indicating a strong inductive effect, whereas a mesomeric effect would increase the electron density at the backbone. This extra positive charge is further compensated by an additional negative charge on the hydrogen atoms and the fluorine atom.

Finally, the influence of an α-fluorine on the activity of Michael acceptor cysteine protease inhibitors was determined using *Leishmania* CPB cysteine protease. Compounds were tested as inhibitors of *L. mexicana* cysteine protease CPB2.8ΔCTE, prepared and assayed as described.[24]

Table 2 shows % inhibition of enzymatic activity at 20 and 2 μg/ml. Assays were carried out in 0.4 ml of 0.1 M sodium acetate, 2 mM EDTA, 1 mM DTT, pH 5.0. Enzyme (5 μg/ml) and inhibitor were added and the mixture was incubated for 10 min at 30 °C. The reaction was started by addition of Z-FR-pNA (300 μM) and the absorbance was monitored at 410 nm for 2 min at 30 °C. The control reaction (no inhibitor) was linear for > 2 min and the rate (change in absorbance at 410 nm) was 1.29×10^{-3}/s. Both isomers **3** were very weak inhibitors. These results confirm the in vitro observations and the theoretical approach, where the introduction of an α-fluorine in a Michael acceptor enzyme inhibitor does not afford biologically interesting compounds.

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