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Research paper

Biological conversion of a water-soluble prodrug of cyclosporine A

F. Lallemand [a], E. Varesio [b], O. Felt-Baeyens [a], Leila Bossy [a], G. Hopfgartner [b], R. Gurny [a,*]

[a] *Department of Pharmaceutics and Biopharmaceutics, University of Geneva, University of Lausanne, Geneva, Switzerland*
[b] *Life Sciences Mass Spectrometry, University of Geneva, University of Lausanne, Geneva, Switzerland*

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Abstract

UNIL088 is a water-soluble prodrug of cyclosporine A (CsA) designed for topical ocular delivery. The pro-moiety is grafted via an ester function to CsA and the solubilizing group is a phosphate ion. The aim of this study was to elucidate the conversion mechanisms by which UNIL088 generates CsA. UNIL088 was incubated in rabbit tears at physiological temperature to study its enzymatic and chemical conversion, respectively. Metabolites and intermediates were identified using a quadrupole-time of flight (QqTOF) mass spectrometer, which allowed biotransformation pathways to be deduced. Conversion is activated by the chemical or enzymatic hydrolysis of the terminal ester function of the pro-moiety, leading to the phospho-serine-sarcosine-cyclosporine A that spontaneously converts into CsA. In addition to the main biotransformation pathway, a secondary reaction involved hydrolysis of the phosphate ester group of the pro-moiety, probably by phosphatases present in tears.

Keywords: Cyclosporine A; Prodrug; Tears; Biotransformation; Enzymes; LC–MS; MS/MS

1. Introduction

Since cyclosporine A (CsA) has shown great potential in the treatment of certain ocular disorders [1], several attempts have been made to topically administer this highly lipophilic molecule. Many delivery systems have been designed for this purpose but none of these systems have been fully satisfactory [2]. The prodrug approach is an effective means of improving the ocular tolerance and effectiveness of poorly water-soluble molecules by chemical derivatization. The most commonly used prodrug strategy for increasing aqueous solubility of drugs containing a hydroxyl group is to produce esters of an ionisable group such as the phosphate group [3]. Such a phosphate grafted onto the hydroxyl function is difficult to hydrolyse by enzymes due to steric hindrance. Hence, the phosphate group must be linked via a spacer group to the parent molecule. Wenger et al. [4] synthesized UNIL088, a water-soluble ester prodrug of CsA. The pro-moiety of this prodrug is linked via a hydroxyl function of the molecule, the solubilizing group is a phosphate ion held by the dipeptide sarcosine-serine-(acyloxy)alkyl-oxy-carbonyl. This molecule has been designed according to the double prodrug theory, i.e., the prodrug is converted into the active parent molecule via a two-step enzymatic and chemical mechanism. The first enzymatic cleavage of the ester group at the end of the pro-moiety releases a chemically unstable compound that spontaneously converts into CsA.

Previous work demonstrated that 20% of UNIL088 was converted into CsA in 30 min in presence of rabbit tears, hydrolysis being mainly enzyme catalysed [5]. A stability study of the prodrug in buffer solutions demonstrated that the hydrolysis of the prodrug followed first order kinetics and allowed partial elucidation of the chemical conversion mechanism [6]. However, it is still unclear whether or not chemical and enzymatic conversion mechanisms are strictly the same. All the intermediates and metabolites released, in particular the pro-moiety must be clearly identified and

* Corresponding author. Department of Pharmaceutics and Biopharmaceutics, University of Geneva, University of Lausanne, Quai E. Ansermet 30, 1211 Geneva 4, Switzerland. Tel.: +41 22 379 61 46; fax: +41 22 379 65 67.
E-mail address: robert.gurny@pharm.unige.ch (R. Gurny).

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characterized before further advancement in the development process of a drug. The aim of this work is to elucidate the chemical and enzymatic conversion mechanisms and to identify the metabolites, using liquid chromatography combined with atmospheric pressure ionisation tandem mass spectrometry (LC–MS/MS). Indeed, the latter analytical technique has proven to be particularly suitable for the analysis of pharmaceutical compounds and their metabolites in biological matrices [7]. Moreover, accurate mass measurements performed on the hybrid MS instrument such as QqTOF improved the confidence in the structural elucidation of the different intermediate species as shown by Hopfgartner et al. [8].

2. Materials and methods

2.1. Materials

UNIL088, phospho-serine-sarcosine-cyclosporine A (pSer-Sar-CsA) and diketopiperazine (DKP) phosphate were synthesized and characterized by the Institute of Chemical Sciences and Engineering (ISIC), Federal Polytechnic School of Lausanne (EPFL), Switzerland. CsA was provided by the ISIC. Phosphate-buffered solution (PBS) was composed of monopotassium phosphate and disodium phosphate (1/15 M). Chemicals of analytical grade were obtained from Fluka Chemie (Buchs, Switzerland). HPLC grade methanol (MeOH) was purchased from Fisher Scientific (Loughborough, UK). Formic acid (FA) was obtained from Merck (Darmstadt, Germany) and water was supplied by a MilliQ Gradient A10 purification unit (Millipore, Bedford, MA, USA).

2.2. Methods

2.2.1. Preparation of prodrug solution

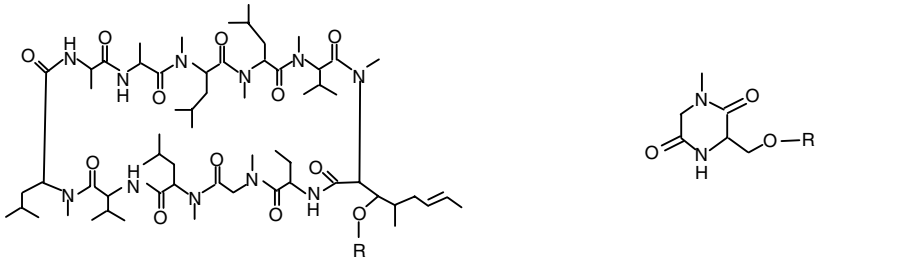
A UNIL088 solution was extemporaneously prepared at a concentration equivalent to 0.2% (w/v) of CsA in an aqueous 1/15 M phosphate buffer solution (PBS) at pH 7.4. The prodrug powder was solubilized at room temperature and under slight mechanical agitation.

2.2.2. Rabbit tear collection

Animal tears were collected from non-anaesthetized New Zealand albino rabbits (weighing approximately 4.0 kg) with disposable 2 μl glass micro-capillaries (Microcaps Drummond, Thomas Scientific, New Jersey). Tear secretion was not artificially stimulated. Immediately after collection, capillaries were blown into a vial under a gentle flow of nitrogen. The biotransformation study of the prodrug into CsA was performed within 5 min after tear collection. The experiments were approved by the local Animal Ethics Committee.

2.2.3. Incubation

A volume of 72 μl of the prodrug solution was incubated under slight agitation with 20 μl of fresh rabbit tears at 37.0 ± 0.5 °C (Thermomixer Comfort, Eppendorf, Hamburg, Germany). An aliquot of 4 μl was collected at pre-determined times (20 time points over a period of 24 h) and mixed with 4 μl of 0.1% (v/v) FA solution in



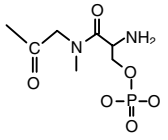
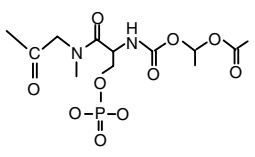
Analyte	Side chain (R)	Analyte	Side chain (R)
Cyclosporine A MW= 1202.64 g/mol $[M+2H]^{2+}$ at *m/z* 601.928	R = H	**DKP phosphate** MW = 238.14 g/mol $[M+H]^{+}$ at *m/z* 239.043	R = PO_3
pSer-Sar-CsA MW = 1440.78 g/mol $[M+2H]^{2+}$ at *m/z* 720.946	R = 	**Diketopiperazine** MW = 158.16 g/mol $[M+H]^{+}$ at *m/z* 159.076	R = H
UNIL088 MW = 1570.88 g/mol $[M+H]^{2+}$ at *m/z* 785.959	R = 		

Fig. 1. Structure of CsA and its derivatives.

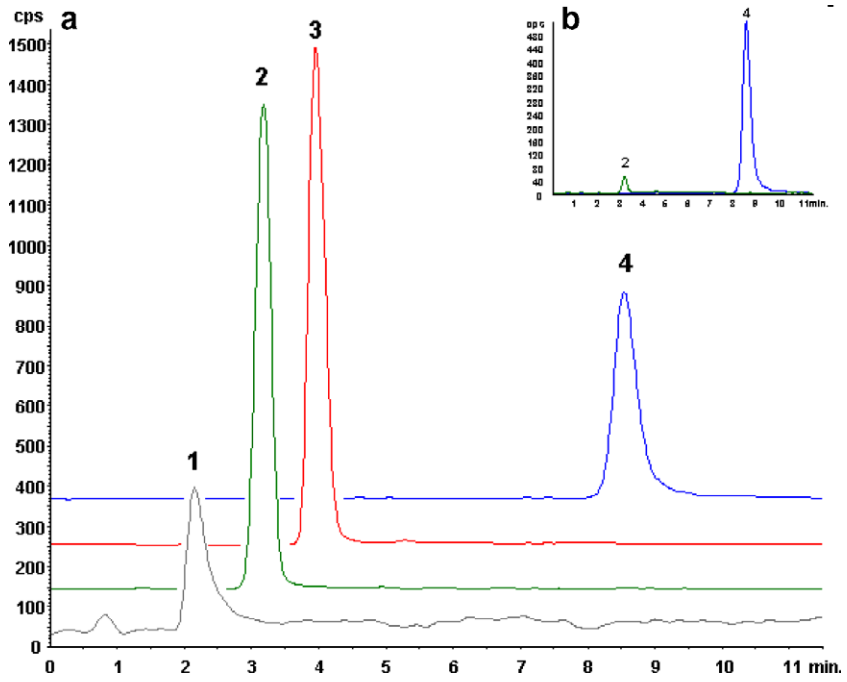


Fig. 2. (a) Extracted ion chromatograms (EIC) of standard solutions (2 μg/ml): DKP phosphate (1), pSer-Sar-CsA (2), CsA (3) and UNIL088 (4). (b) EIC of the UNIL088 standard solution for pSer-Sar-CsA and UNIL088 doubly charged ions.

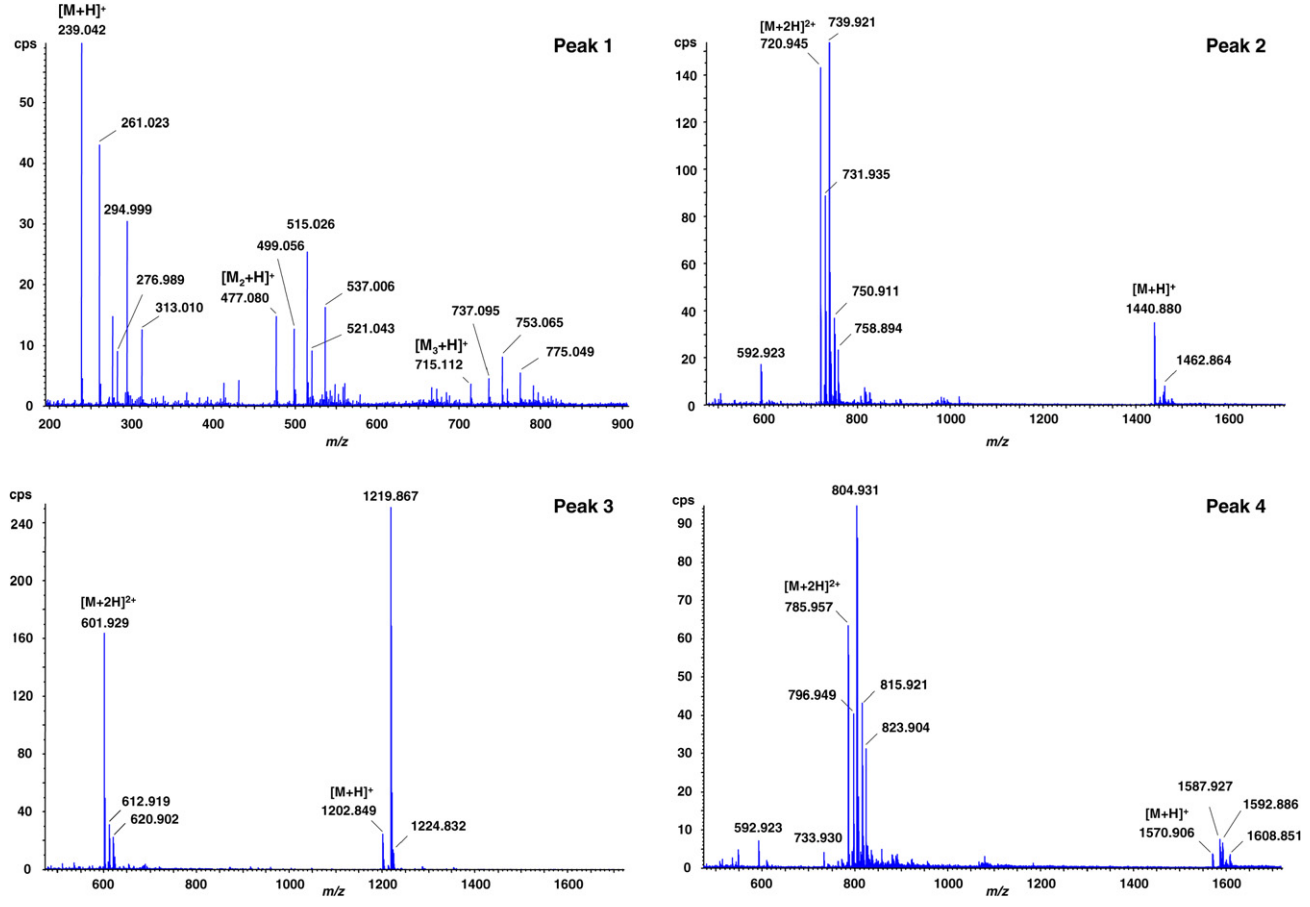


Fig. 3. TOF MS spectra of standard solution LC–MS analyses. Peaks are labelled according to Fig. 2.

water–MeOH (1:1, v/v) on ice in order to quench the biotransformation reaction. Sample was then vortex-mixed and centrifuged at 9000 rpm (6792 rcf) for 3 min at 4 °C. A 50-fold dilution was performed by mixing the supernatant with a solution of 0.1% (v/v) FA in water–MeOH (1:1, v/v). This dilution step was performed in order to avoid microcolumn overloading during LC–MS analysis.

2.2.4. LC–MS

Compounds were analysed by high-performance liquid chromatography (HPLC) coupled with a QqTOF mass spectrometer. The HPLC system consisted of two binary gradient pumps (model LC-10ADvp), a degasser (model DGU-14A), a column oven (model CTO-10ASvp) and an autosampler (model SilHTc) from Shimadzu (Kyoto, Japan). Separation was performed on a C8 Symmetry Shield microcolumn (2 mm I.D. × 5 cm–5 μm/100 Å) from Waters (Milford, MA, USA). Aqueous mobile phase (phase A) consisted of 0.1% (v/v) FA in water and organic mobile phase (phase B) consisted of 0.1% (v/v) FA in methanol. A linear gradient from 70% B to 100% B in 3 min followed by a washing step of 3 min before column re-equilibration was employed. The flow rate was set at 200 μl/min and the column oven was set at 40 °C. The overall analysis run time was 12 min. Samples were kept at 10 °C in the autosampler before injection (50 μl).

MS analysis was performed on a QSTAR XL mass spectrometer (AB/Sciex, Concord, ON, Canada) equipped with a TurboIonSpray interface heated at 100 °C. The sprayer voltage was set at 5500 V (positive polarity) and the nebulizing gas was nitrogen. For TOF-MS experiments, the mass range was of m/z 100 to m/z 2000 and spectra were collected for 1 s. For MS/MS experiments, the mass range was the same as for MS experiments and Q1 resolution was set to 0.7 Da (FWHM). The collision gas was nitrogen and the collision energy was set to 35 eV (laboratory frame). Nitrogen was provided by a Domnik Hunter nitrogen generator (model UHPLCMS25, Durham, UK).

3. Results

Individual standard solutions of CsA, UNIL088 and its probable metabolites, pSer-Sar-CsA and DKP phosphate (Fig. 1) were injected and analysed by LC–MS. As shown in Fig. 2a, all the compounds were separated in less than 10 min. All the CsA derivatives (i.e. pSer-Sar-CsA, CsA and UNIL088) had typical MS spectra with different salt adducts for both the mono- and doubly charged pseudo-molecular ions (Fig. 3 – peaks 2–4, respectively). However, DKP phosphate with a molecular weight of 238.14 Da showed multimeric pseudo-molecular ions also with different salt adducts such as sodium or potassium (Fig. 3 – peak 1).

As shown in Fig. 2b, UNIL088 standard contains approximately 2–4% of pSer-Sar-CsA. Thus, when a 0.2% (w/v) UNIL088 solution was extemporaneously prepared and incubated at 37 °C with rabbit tears, the concentration of pSer-Sar-CsA was significant at the beginning of the biotransformation study. Nevertheless, the concentration of pSer-Sar-CsA decreased very rapidly during the first 15 min (estimated half-life of 10 min) and was converted into CsA. Then, its concentration reached a plateau and remained relatively constant during the entire incubation study, indicating that the biotransformation of the prodrug into CsA via the generation of pSer-Sar-CsA intermediate had reached equilibrium. Thus, CsA was generated quantitatively following a first-order kinetic conversion rate. It is worth mentioning that a compound with a pseudo-molecular ion of m/z 742.935 (i.e. a doubly charged ion) co-eluted with the pSer-Sar-CsA analyte. With a mass accuracy of less than 10 ppm this intermediate might correspond to the carbamic acid derivative of pSer-Sar-CsA, but further investigations are needed for an unambiguous structural confirmation.

Fig. 4 shows typical chromatograms of tear samples incubated with the prodrug solution after 2 min and

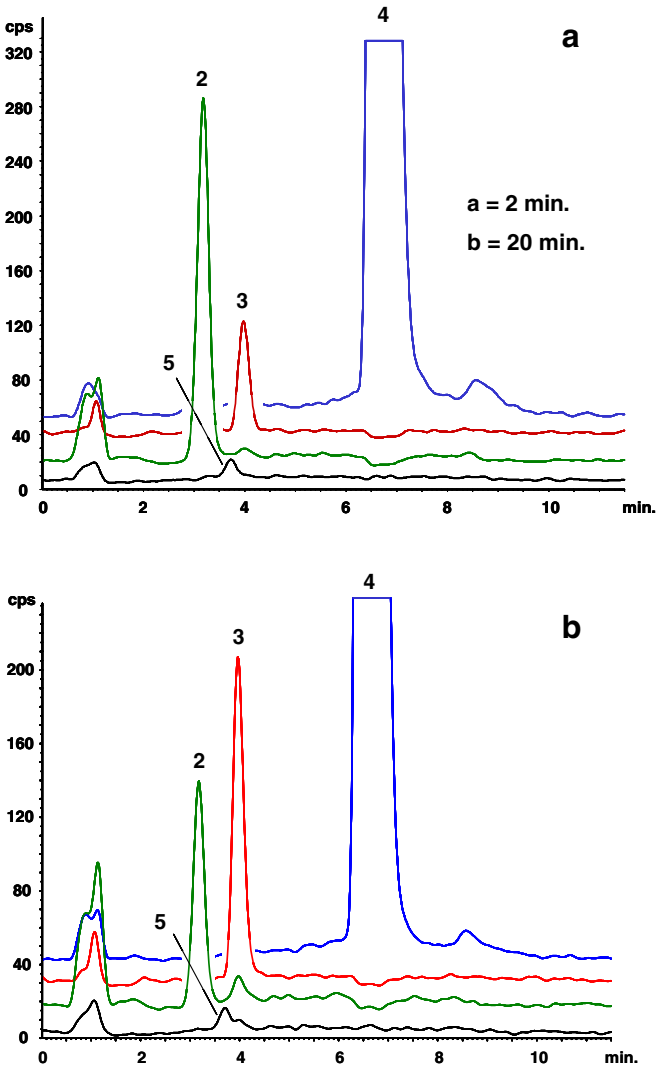


Fig. 4. EIC traces of pSer-Sar-CsA (2), CsA (3), UNIL088 (4) and RT37 (5) for the LC–MS analysis of UNIL088 biotransformation in rabbit tears. (a) Incubation time of 2 min, (b) Incubation time of 20 min.

20 min of incubation at 37 °C. As expected, the concentration in CsA increased over time, but the conversion of pSer-Sar-CsA into DKP or DKP phosphate was not observed. No other compounds, such as the pSer-Sar and Ser-Sar dipeptides or other related species, were detected when performing extracted ion chromatograms.

Another CsA derivative was observed at around 3.7 min suggesting a second conversion pathway of UNIL088 (Fig. 4 – peak 5). A mass spectrum of this compound revealed a singly charged ion at *m*/*z* 1490.944 for the pseudo-molecular ion and a doubly charged ion at *m*/*z* 745.984 (Fig. 5). Therefore, MS/MS analysis was performed in order to determine whether this derivative possessed a similar fragmentation pattern to CsA derivatives. By observing the low mass region of the MS/MS spectra, some marker ions from the CsA backbone, such as the immonium ion of 2-aminobutyric acid at *m*/*z* 58.1, the immonium ion of *N*-methyl valine at *m*/*z* 86.1, the immonium ion of *N*-methyl leucine at *m*/*z* 100.1 or a fragment at *m*/*z* 156.1 corresponding to the formylated *N*-methyl leucine, could be identified. Together, these findings suggested that the new CsA derivative corresponded to the dephosphorylated UNIL088 prodrug. Further investigations have to be carried out to confirm these findings.

4. Discussion

The LC–MS method employed in this study has enabled the identification of UNIL088 metabolites. Hamel et al. [9] described the theoretical conversion mechanism leading to the formation of CsA and DKP phosphate in a two-phase reaction via the pSer-Sar-CsA intermediate. In this latter article [9], only one transformation pathway was described. The main biotransformation pathway of UNIL088 into CsA described in a previous article [6] and confirmed by this study (pathway 1 – Fig. 6) consists of the hydrolysis of the terminal ester of the (acyloxy)alkyl-oxy-carbonyl group leading to a reaction cascade of intramolecular hydrolysis and release of CsA via the pSer-Sar-CsA intermediate in the presence of tear fluid by an esterase-like activity of the tear proteins [5]. The conversion of pSer-Sar-CsA into CsA is a very rapid step as shown by the disappearance of this intermediate over the first 15 min of the biotransformation study. The exact mechanism of this conversion step is not confirmed yet and remains hypothetical: the terminal amine of the pSer-Sar-CsA should attack the ester connecting the pro-moiety to CsA via an addition-elimination reaction. Hamel et al. [9] assert that the release of CsA is accompanied by a subsequent release of DKP phosphate by cyclization of the pSer-Sar dipeptide. In the present study neither the pSer-Sar or Ser-Sar dipeptides, nor any structurally related compounds, were isolated or identified so far.

The loss of the phosphate group of UNIL088 demonstrates the presence of an additional biotransformation pathway (Pathway 2 in Fig. 6). In vitro hydrolysis of the phosphate monoester was reported to be 10–1000 times slower relative to that in the presence of human phosphatases [10] [3]. The high dephosphorylation rate of ester phosphate in the presence of rabbit tears is consistent with the presence of lysosomal enzymes, in particular acid [11] and alkaline phosphatases [12]. This observation is confirmed by other authors: Polansky and Weinreb [13] suggested that topically administered dexamethasone phosphate in eye drops may be hydrolysed into dexamethasone by phosphatases; a hypothesis confirmed later by

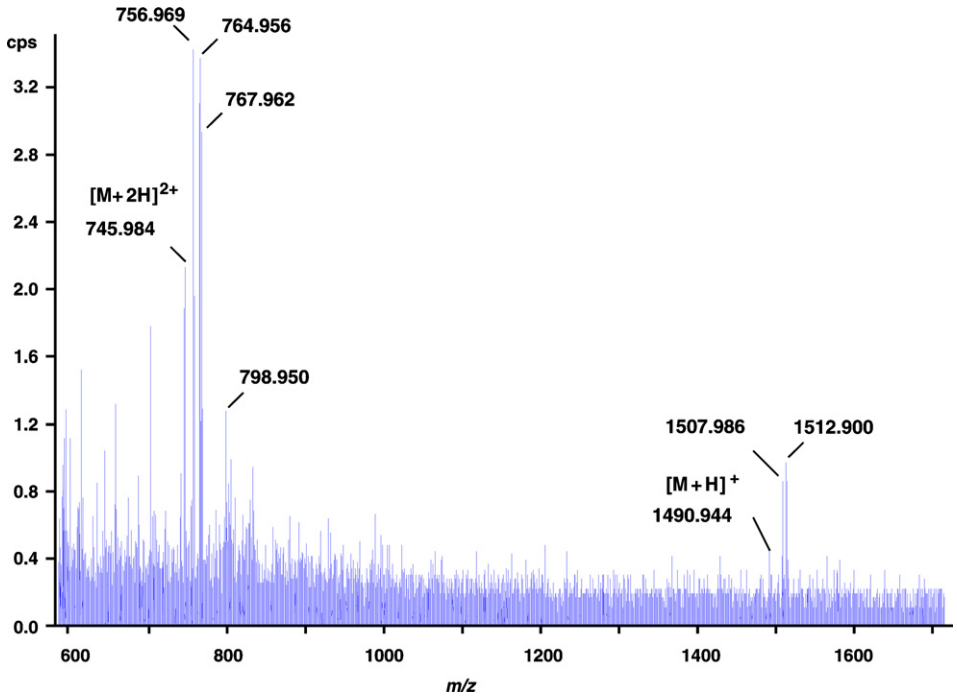


Fig. 5. TOF MS spectrum of compound eluting at 3.7 min in Fig. 4.

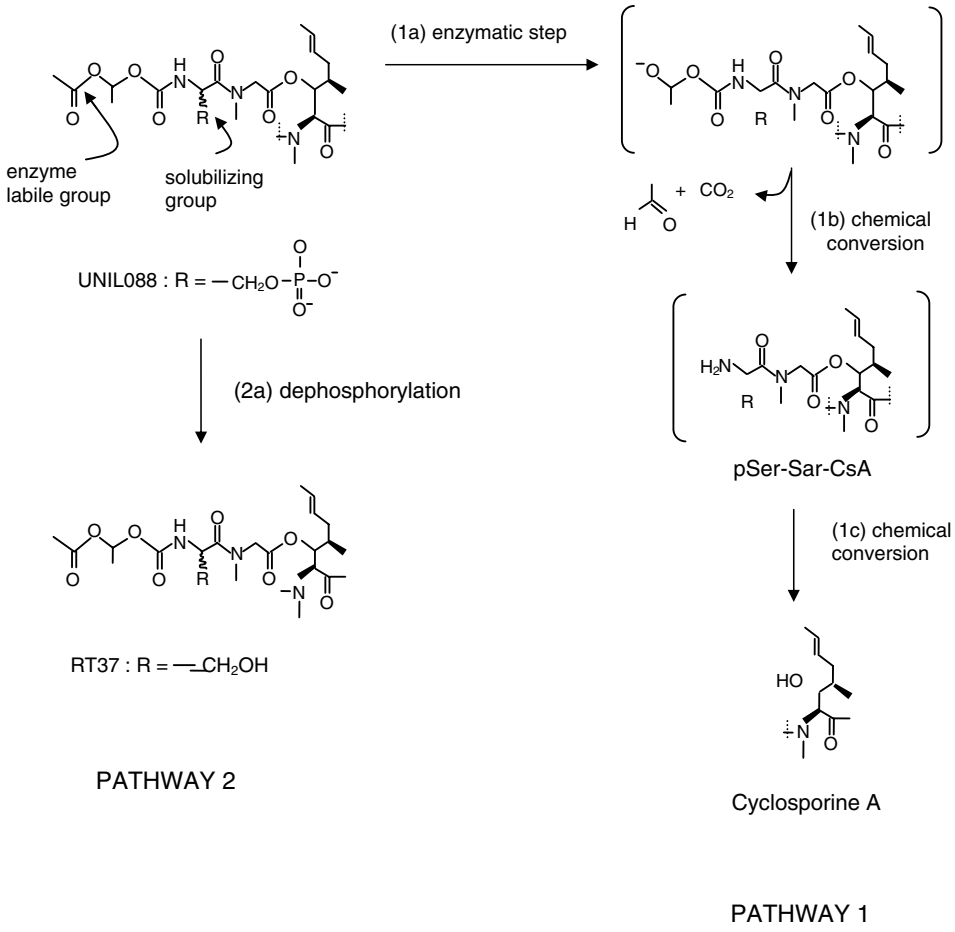


Fig. 6. Proposed pathways for the biotransformation of UNIL088 into cyclosporine A. See text for details.

Baeyens et al. who observed that released dexamethasone phosphate from an insert in the precorneal area was cleaved in the tear fluid into dexamethasone [14]. The presence of this enzyme in the tears and eye tissues is already exploited to convert phosphate ester prodrugs of prednisolone [15].

5. Conclusion

The chemical and enzymatic conversion mechanisms of UNIL088 are obviously identical, although their kinetics have been shown to be different with enzymatic biotransformation being more rapid than chemical conversion [6]. The main pathway of transformation of UNIL088 into CsA is confirmed by this study. The dipeptide pSer-Sar released from the pro-moiety was not identified and the fate of this fragment remains to be investigated. The incubations showed a second metabolic pathway consisting of the hydrolysis of the ester phosphate of UNIL088. This hydrolysis is catalysed by the phosphatases present in the tears. This metabolic route releases a compound that does not appear to be converted into CsA, however, the accumulation observed in vitro is not expected to occur in vivo since the normal tear turn over is very rapid. The exact contribution of this pathway in the in vivo metabolism of UNIL088 remains to be investigated.

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