Cyclization reactions of IMM-125 and oxidation of cyclosporin A amino-acid 1 in the \( \alpha \) position of the double bond lead to the loss of \textit{in vitro} immunosuppressive activity

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\textit{Dedicated to the memory of Dr. Piet Leclercq}

\textbf{Abstract.} Cyclosporin A (CsA) and IMM-125, a hydroxyethyl derivative of D-serine CsA, are cyclic undecapeptides of molecular weight 1201.8 and 1261.8, respectively. The main metabolites still possessing the undecapeptide structure were found to be compounds resulting from the biotransformation of amino acids 4, 9 and 1. Under the influence of the hepatic cytochrome P-450-dependent monooxygenase system, CsA and IMM-125 amino acid 1 are metabolized to a mono-hydroxylated compound (metabolite M-17) and to a dihydrodiol. A metabolite M18 was found to be the result of a non-enzymic intramolecular formation of a tetrahydrofuran derivative from metabolite M17. Since the existence of a CsA dihydrodiol was reported and since epoxides are considered as the dihydrodiol precursors, the aim of the present work was to prove that the same non-enzymic intramolecular formation of a tetrahydrofuran ring could occur by nucleophilic attack of the amino-acid 1 \( \beta \)-hydroxy group at the \( \varepsilon \)-position of the freshly formed epoxide by reaction of IMM-125 with m-chloroperbenzoic acid and cyclosporin A with selenium oxide. The immunosuppressive activity of the compounds, as measured by the mixed lymphocyte reaction and by the luciferase activity of a Jurkat-T-cell line stably transfected with the NF-AT/luc reporter plasmid, was found negligible for IMM-125 compared to the parent drug as well as for the cyclosporin A derivative. Structures of the IMM-125 and CsA derivatives were elucidated by electrospray mass-spectrometry and NMR spectroscopy.

Keywords: Immunosuppressive drugs, nuclear magnetic resonance spectroscopy, mass spectrometry, high performance liquid chromatography

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1. Introduction

Cyclosporin A (CsA) is a cyclic undecapeptide (Fig. 1) of molecular weight 1202.6 (C_{62}H_{111}N_{11}O_{12}) isolated from Rypocladium inflatum and widely used to prevent graft rejection after organ transplantation (kidney, heart, lung, skin, bone marrow, etc.) and more recently to treat psoriasis and a number of autoimmune diseases (diabetes, nephrotic syndrome, etc.). IMM-125, a hydroxyethyl derivative of D-serine CsA, has a molecular weight of 1261.85 (C_{64}H_{115}N_{11}O_{4}) and has been shown to be equipotent to CsA in many in vitro test systems and to have a similar ability to suppress lymphokine production [1].

Research concerning CsA and IMM-125 metabolism has been mainly focused on the hepatic cytochrome P-450-dependent monooxygenase system [2–8] but recently it was shown that after oral administration approximately two-thirds of the cyclosporin metabolism occurs in the gut, the liver being responsible for one third of the metabolism [9–11].

The main metabolites which were identified still possess the undecapeptide structure and were mainly found to be compounds resulting from the biotransformation of amino-acid 4 (metabolite M21, N-demethylation), amino acid 9 (metabolite M1, hydroxylation) and amino acid 1 (metabolites M17, M18 and a dihydrodiol) as reported by Maurer [2] and Lhoëst [8,12]. Metabolite M18 was found to be the result of a non-enzymatic intramolecular formation of a tetrahydrofuran derivative from metabolite M17. Since the existence of a CsA dihydrodiol was reported and since epoxides are considered as the dihydrodiol precursors, the aim of the present work was to synthesize a transitory epoxide in order to prove that the same non-enzymic intramolecular formation of a tetrahydrofuran ring occurs by nucleophilic attack of the hydroxy group at the ε-position of the freshly formed epoxide by reaction of CsA or IMM-125 with m-chloro-perbenzoic acid. Moreover a peculiar derivative of cyclosporin A retaining the in vitro immunosuppressive activity of cyclosporin A and resulting from the oxidation of CsA amino acid 1 has been identified by electrospray mass spectrometry and NMR spectroscopy.

The in vitro immunosuppressive activity of these IMM-125 and CsA derivatives will also be discussed.

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![Fig. 1. Structure of IMM-125 (top) and CsA (bottom).](image-url)
2. Material and methods

2.1. Chemicals and reagents

CsA and IMM-125 were kindly supplied by Novartis Pharma (Basle, Switzerland). Spectrograde solvents such as methanol, acetonitrile and dichloromethane used in extraction or analytical procedures were purchased from Labscans Ltd. Unit T26 (Dublin, Ireland) and hexane from Alltech Associates Sciences Laboratories (Deerfield, IL, USA). Meta-chloroperbenzoic acid was purchased from Sigma-Aldrich (Bornem, Belgium).

2.2. Synthesis of an IMM-125 amino acid 1 derivative

m-Chloroperbenzoic acid (2.4 mg) was added to a chloroformic solution (1.5 ml) of CsA or IMM-125 (1.74 mg) and the mixture was kept at 0°C and magnetically stirred for 1 hour (molar ratio 7:1). After addition of 2 ml of water acidified with acetic acid (pH = 4), the mixture was vortexed for 3 minutes and transferred to a separatory funnel. The organic phase was collected in a conical bottom flask and evaporated under reduced pressure. The dry residues were then submitted to HPLC analysis.

2.3. Synthesis of a CsA amino acid 1 derivative

CsA (1.8 mmol) dissolved in 500 μl dichloromethane were put into reaction with 18 mmol of SeO₂ suspended in 500 μl of dichloromethane. The mixture was left at room temperature under stirring for 18 hours. The solvent in the reaction mixture was evaporated under a stream of nitrogen. Acetic acid (100 μl) was added to the dry residue and this mixture was left for 2 h at 30°C. To this mixture cooled in ice was added 2 ml of water and 600 μl of a K₂CO₃ aqueous solution at 20% (pH 7.4). The mixture was then extracted two times with 3 ml dichloromethane and the organic phase was evaporated to dryness under reduced pressure for further HPLC analysis.

2.4. HPLC

The HPLC system consisted of 2 LC10AD Shimadzu pumps, a Waters (Brussels, Belgium) U6K injector and 2487 dual absorbance LC-UV detector connected to an AST computer loaded with a Softron PC integration pack (Kontron, Switzerland). The residues were chromatographed on a Waters Novapak C18 column (6 μm, length 300 mm, i.d. 7.8 mm). The mobile phase was a mixture of acetonitrile/water (60/40 or 52/48), and the flow rate and UV detector settings were 2.5 ml/min and 212 nm, respectively; the oven temperature was 50°C. Under those conditions two peaks were collected for IMM-125 at retention times of 8.3 minutes (RT8) and 15 minutes (RT15) and RT10.7 for CsA. After evaporation of the mobile phase under reduced pressure, the residues were dissolved in acetonitrile, transferred to individual tubes preweighed on a semimicro balance (Precisia, Zürich, Switzerland) and evaporated to dryness under a stream of nitrogen. A quantity of 800 μg was accumulated to obtain sufficient amounts for evaluation of its in vitro immunosuppressive activity, for NMR and for electrospray mass spectrometry.

2.5. Electrospray-MS/MS

Electrospray MS spectra were obtained with a Finnigan LCQ MSn instrument (San Jose, CA, USA). The source voltage was 5.0 kV, the capillary voltage 25.7 V, and the capillary temperature was 190°C.
The octopole offsets 1 and 2 were set to 5.5 and 3.0 V and the flow of dry gas (N₂) was 8 l/min. The compounds (50 µg) were dissolved in a mixture of acetonitrile/5 mM aqueous solution of ammonium acetate (analyte concentration 10 ng/µl) and the solution was infused with the aid of a syringe pump at a flow rate of 5 µl/min.

2.6. Electrospray-MS

Electrospray ionization (ESI) mass spectra were obtained with a Jeol Lcmate benchtop LC/MS system (JEOL LTD, Tokyo, Japan). The source voltage was set at 2.5 kV, the ESI/needle voltage at 1.95 kV, the needle current was 1.9 µA, the desolvation plate temperature was 200°C, the orifice, ring lens and ion guide were set at 93 V, 207 V and 2.9 V, respectively, and the flow rate of dry gas (N₂) was 7 l/min. The compounds (50 µg) were dissolved in a mixture of acetonitrile–7.5 mM ammonium acetate (50 : 50) and the solution was infused with the aid of a syringe pump at a flow-rate of 10 µl/min.

2.7. NMR

1H NMR spectra of the IMM-125 derivatives RT8 and RT15 (1 mg) were recorded in CDCl₃ at 300 K on a Bruker DRX-400 or AMX-500 spectrometers (Bruker, Rheinstetten, Germany), equipped with a Silicon Graphics data station. Spectra were referenced relative to the residual solvent peak (CHCl₃, δ = 7.24 ppm).

Measurement conditions. The 1D 1H spectra were acquired in overnight runs (6k scans) with 64 k data points over a sweep width of 10204 Hz, using a 30 degree pulse, a 3 s relaxation delay and quadrature detection. Prior to Fourier transform, data was zero filled up to 64 k real points and apodized with a 0.3 Hz exponential decay window function.

Phase-sensitive double quantum-filtered 1H correlation spectra (DQF-COSY) [13] were obtained with the sequence $D_1-90°-t_1-90°-t_2$. 256 Experiments in $t_1$ with 128 transients each were acquired using a relaxation delay $D_1$ 1.5 s, a delay $t$ of 3 µs, a 90° pulse 9.2 µs, 2k data points in $t_2$, a sweep width 5050 Hz in $F_1$ and $F_2$, quadrature detection in $F_1$ and in $F_2$, zero filling up to 2k in $F_1$ and apodization with a π/2-shifted sine bell in both dimensions.

1H total correlation spectra (TOCSY) [14] were acquired using the sequence $D_1-90°-t_1-\text{trim}$-$\text{MLEV17}$-$\text{trim}$-$t_2$ with a relaxation delay $D_1$ 1 s, a 90° pulse 9.0 µs for hard pulses, 2.5 ms for trim pulse and 26 µs for MLEV17 ((90°-180°-90°)₁₆-60°)$ⁿ$ a mixing time 65 ms, 2k data points in $t_2$, a sweep width 4762 Hz in $F_1$ and $F_2$, 236 experiments in $t_1$ with 256 transients each, quadrature detection in both dimensions, zero filling up to 1k in F1 and apodization with a π/2-shifted sine bell in both dimensions.

1H rotating frame nuclear Overhauser effect spectra (ROESY) [15] were obtained using the sequence $D_1-90°-t_1$-Spin lock-$t_2$ with a relaxation delay $D_1$ of 1.5 s, a 90° pulse of 9.0 µs, a mixing time of 250 ms, 2k data points in $t_2$, a sweep width of 4762 Hz in $F_1$ and $F_2$, 256 experiments in $t_1$ with 128 transients each, quadrature detection in both dimensions, zero filling up to 1k in F1 and apodization with a π/2-shifted squared sine bell in both dimensions.

Inverse detected 1H/$^{13}$C heteronuclear multiple quantum correlation spectra (HMQC) [16] were acquired using the sequence $D_1-90°$-$t_1$-$\text{Spin}$-$t_2$-$\text{MLEV17}$-$t_1$-$90°$-$t_2$ with a relaxation delay $D_1$ of 1 s, a presaturation delay $D_2$ of 419 ms, an evolution delay $\Delta_1$ of 3.7 ms, 90° pulses of 9.0 µs for $1\text{H}$ and 8.8 µs for hard $^{13}\text{C}$ pulses, GARP decoupling during acquisition, 2k data points in $t_2$, a sweep width of 4762 Hz in $F_2$ and of 21381 Hz in
Fig. 2. ESI+ mass spectrum of compound RT15.

$F_2$, 298 experiments in $t_1$ with 512 transients each, quadrature detection in both dimensions, zero filling up to 0.5k in F1 and apodization with a $\pi/2$-shifted sine bell in both dimensions.

3. Imunosuppression assay

3.1. MLR reaction

Mononuclear cells were isolated from human peripheral blood by density gradient centrifugation on Ficoll–Hypaque medium (International Medical S.A., Belgium, $d = 1.077$). After washing, the cells were suspended in the enriched Roswell Park Memorial Institute (RPMI) medium at a concentration of $2 \times 10^6$ cells/ml. The enriched RPMI medium consisted of 77% RPMI medium n°1640 (Gibco Ltd. Paisley, Scotland), 20% fetal calf serum (Biosys S.A., France), 1% glutamine, 1% peni-streptomycin (5000 U/ml) and 1% gentamicin (50 mg/ml). $10^5$ cells/well (50 $\mu$l) were incubated in microplates at 37°C, 5% CO$_2$ for 5 days with 50 $\mu$l of the metabolites solution (concentrations were 0.1, 1, 10, 100, 250, 5000, 1000, 2500, 5000 ng/ml in RPMI containing 0.082% of acetonitrile), $10^5$ non-irradiated MHC incompatible allogenic cells (50 $\mu$l) and 50 $\mu$l of enriched medium. 10 $\mu$l of a $^3$H thymidine solution
(0.2 mCi/ml, Isotopchim, Ganagobie, Peyruis, France) was added to each well. Cell cultures were harvested using an automated multiwell harvester (Skatron, Lier, Norway) that aspirates cells, lyses cells and transfers DNA onto filter paper, while allowing unincorporated \(^3\)H thymidine to wash out. The incorporation was determined by liquid scintillation counting (\(\beta\) counter cpm, Veckman LS 6000SE, Breaville, USA) after an additional 8 h of incubation. The potential inhibitory response of each solution was calculated in cpm and expressed as the percentage of inhibition of a normal response (MLR or mixed lymphocytes reaction performed in absence of any solution). Each culture, including positive and negative controls, was performed in triplicate, and all experiments were repeated two times. Addition of pure IMM-125 or CsA and solvent were the positive and negative controls, respectively.

3.2. Jurkat T-cell line

A Jurkat T-cell line stably transfected with a luciferase reporter driven by the NF-AT transcription factor [17] was used to assay the immunosuppressive activity of the CsA analogues. Cells were grown in RPMI medium supplemented with 10% fetal calf serum (HyClone, Logan, UT) and penicillin (200 U/ml). 500,000 cells per sample were plated in 12-wells plates (1 ml) and activated with a combination of PMA (100 ng/ml) and A23187 (500 ng/ml).
Alternatively, they were activated by TCR/CD3 cross-linking using the anti-CD3 mAb OKT3, affinity purified from hybridoma supernatant using sepharose-conjugated protein G (Mabtrap, Pharmacia Biotech, Italy). OKT3 cross-linking was carried out by precoating the plastic well with secondary anti-mouse IgG as described [18]. Immunosuppressants were added 30 min before activations, at final concentrations ranging from 1000 ng/ml to 1 ng/ml. After a 6 hr incubation, cells were collected and assayed for luciferase activity as described [17]. Negative controls included each immunosuppressant at 1000 ng/ml. Experiments were repeated two times, each with duplicate samples.
4. Results and discussion

The electrospray mass spectrum IMM-125 oxidation compound RT15 (Fig. 2) reveals the presence of molecular adducts of mass $m/z = 1279$ $(M + H)^+$ and $1301$ $(M + Na)^+$. This proves that one oxygen was introduced into the cyclosporin A molecule during the oxidation process with m-chloro perbenzoic acid.

The product ion spectrum of the sodium adduct at $m/z = 1301$ (Fig. 3) showed relevant fragment ions of $m/z = 1283$ $(M – O + Na)^+$, $1273$ $(M – CH_2=CH_2 + Na)^+$, $1255$ $(M – CH_3–H_2–OH + Na)^+$, $1239$ $(M – HO–CH_2–CH_2–OH + Na)^+$, as illustrated in the fragmentation pathway of Fig. 4. This indicates that IMM-125 was oxidized to a transient epoxide which was directly submitted to a nucleophilic addition of the hydroxy group located in the β-position of the IMM-125 amino acid to produce a substituted tetrahydrofuran ring.

Compound RT8 exhibits the same electrospray mass spectrum and practically the same product ion spectrum as those of compound RT15 so that the presence of diastereoisomeric compounds could be suspected as demonstrated by nuclear magnetic resonance spectroscopy (NMR).

The assignments of the $^1$H-NMR spectra of the reaction products for IMM-125 (Fig. 5) have been carried out using a combination of homo- and heteronuclear 2D-NMR techniques (double quantum-
The chemical shifts are: 3.30, 3.26, 3.24, 3.03, 2.79, 2.70 and 2.66 ppm.

In the HMQC NMR spectra of compounds RT8 and RT15 the signals corresponding to the ethylenic carbon atoms have been replaced by two supplementary signals in the $\delta^{(13)C} = 58–82$ ppm region. These supplementary signals at 64 and 79 ppm for RT 8 and at 83 ppm for RT15 could be assigned to the $\varepsilon$- and $\zeta$-protons, respectively. In both cases the $\beta$-carbons have been deshielded by $\sim 10$ ppm. Therefore, the $^{13}C$ chemical shift data correspond to a free hydroxyl function in the $\zeta$-position and to ether-type

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CH$_3$ groups have not been univoquely assigned.

Chemical shifts are: 3.30, 3.26, 3.24, 3.03, 2.79, 2.70 and 2.66 ppm.

filtered COSY, TOCSY, ROESY and HMQC) and were ascertained by comparison with the previously reported assignments for IMM-125 [7]. $^1$H chemical shift data for the IMM-125 derivatives are gathered in Table 1.

In the HMQC NMR spectra of compounds RT8 and RT15 the signals corresponding to the ethylenic carbon atoms have been replaced by two supplementary signals in the $\delta^{(13)C} = 58–82$ ppm region. These supplementary signals at 64 and 79 ppm for RT 8 and at 83 ppm for RT15 could be assigned to the $\varepsilon$- and $\zeta$-protons, respectively. In both cases the $\beta$-carbons have been deshielded by $\sim 10$ ppm. Therefore, the $^{13}C$ chemical shift data correspond to a free hydroxyl function in the $\zeta$-position and to ether-type
Fig. 6. Remarkable ROe’s observed for RT8 (left) and RT15 (right).

Fig. 7. Compared ESI+ mass spectra of CsA oxidation compound RT10.7 (top) and CsA (bottom).
Stereochemistries of the cyclisation compounds have been determined using ROESY-spectroscopy. The ROESY spectrum of compound RT8 reveals dipolar interaction between the $\alpha-\varepsilon$, $\beta-\delta$, $\gamma-\delta$ and $\gamma-\varepsilon$ protons which agree with the structure represented in Fig. 6, i.e., with absolute configurations R and $\beta$- and $\varepsilon$-carbons. Together with the data from ESI-MS-spectrometry, this suggests the opening of an intermediary epoxide by an intraresidual reaction with the hydroxyl function in position $\beta$.

Fig. 8. Fragmentation pathways of compound RT10.7.

Fig. 9. Immunosuppressive activity of compounds RT8 and RT15 compared to CsA as determined by the mixed lymphocyte reaction (MLR).
Fig. 10. Luciferase activity in duplicate samples of a Jurkat T-cell line stably transfected with the NF-AT/luc reporter plasmid. Cells were activated either with a combination of PMA and A13187 (upper panel) or with anti-CD3 mAb (lower panel). For each graph the mean value for the activated sample (control) was taken as 100% and the treated samples were calculated as % control. CsA compounds (RT8 and RT15) concentrations are expressed as ng/ml, with a range of concentrations spanning 1000–10 ng/ml. The luciferase values for the PMA/A23187 experiment were 6.7 ± 3.0 relative luciferase units for non activated cells and 849.1 ± 85.0 for activated cells; for the anti-CD3 mAb experiment 1.5 ± 0.15 relative luciferase units for non activated cells and 123.7 ± 9.5 for activated cells.

S for the ε and ζ groups, respectively, one of the two possible furanes, resulting from intramolecular ring-opening of a transient epoxide.

For RT15 the dipolar interactions between H_ε and H_γ, as well as the one between H_α and H_ε have been replaced by another one between H_ε and H_ζ and γ-CH_3 and H_ε, thus agreeing with the structure corresponding to the furane resulting from the ring opening of the isomeric epoxide leading to the formation of RT8 (Fig. 6), i.e., with absolute configurations S and R for the ε and ζ groups, respectively.
Fig. 11. Immunosuppressive activity of compounds RT10.7 compared to CsA as determined by the mixed lymphocyte reaction (MLR).

The electrospray mass spectrum (Fig. 7) of an oxidation product (RT10.7) of cyclosporin A by selenium oxide reveals the presence of molecular adducts of mass $m/z = 1235 (M + H)^+,$ $1257 (M + Na)^+,$ $1273 (M + K)^+$, as well as fragmentation ions of mass $m/z = 1241 (M − O + Na)^+,$ $1223 (M − O − H_2O + Na)^+,$ $1217 (M − 2H_2O)H^+,$ $1091 (M − 144)H^+,$ indicating that CsA amino acid 1 was classically oxidized by selenium oxide in the $\alpha$ position of the double bond as illustrated in the fragmentation pathways of Fig. 8.

The immunosuppressive activity of compounds RT8 and RT15, as measured by the mixed lymphocyte reaction (Fig. 9) and by the luciferase activity of a Jurkat T-cell line stably transfected with the NF-AT/luc reporter plasmid (Fig. 10), was found negligible compared to the parent drug. The same results were observed (Fig. 11) for an oxidation product of CsA (RT10.5). Since it has been reported [19], that in the non covalent complex between CsA and cyclophilin A (CyPA), the $\beta$-hydroxy group of CsA amino acid 1 is engaged in a direct hydrogen bond with the CO group of methylleucine 4, the observed loss of immunosuppressive activity is most probably due to the modification of the types of interactions between OH groups of CsA amino acid 1 and specific amino acids of cyclophilin A decreasing the binding affinity of the drug-CyPA binary complex.

References

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