First chemoenzymatic synthesis of immunomodulating macrolactam pimecrolimus

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Abstract
The preparation of pimecrolimus, a synthetic derivative of ascomycin endowed with immunomodulatory activity, requires the selective protection of 24-hydroxy group of the ascomycin, before elaboration of the 32-hydroxy group. The aim was achieved by means of two regioselective Candida antarctica lipase-catalyzed steps. The structure of the new key intermediates, 24-, 32-monoacetates, and 24,32-diacetate, was established by means of an unambiguous NMR study.

1. Introduction
The ascomycin derivative pimecrolimus 1 (32-chloro-32-epiascomycin, present on the market with the trade name of Elidel®) is an immunomodulating 23-membered macrolactam approved for topical treatment of inflammatory skin diseases such as atopic dermatitis. Its pharmacological activity is due to the formation of a complex between the macrolactam and an immunophilin that inhibits calcineurin, the phosphatase involved in T-cell activation and inflammatory cytokines synthesis.

Ascomycin 2, isolated from fermentation broth of the soil fungus Streptomyces hygroscopicus, is converted into pimecrolimus 1 by substitution of 32-hydroxy group with a chlorine with inversion of configuration.

The contemporary presence of two hydroxy groups, at positions 24 and 32, requires, before introduction of the chlorine, the protection of the 24-alcohol, usually carried out by silylation of both hydroxy groups, to give 3, followed by deprotection at position 32. Large amounts of silylating agent (TBDMSCl or TBDMOSiCl) are required and purification by column chromatography is necessary both after the silylation and the deprotection step, affording intermediate 4 in 84% yields.

2. Results and discussion
2.1. Chemoenzymatic approach to the synthesis of pimecrolimus 1
The known selectivity of lipases prompted us to study a chemoenzymatic approach to the preparation of a suitably 24-protected intermediate. A screening among the commercially available lipases, in irreversible transesterification conditions, allowed us to conclude that ascomycin 2 was not substrate for Pseudomonas fluorescens (PFL), porcine...
pancreas (PPL), and Candida cylindracea (CCL) lipases, whereas Candida antarctica lipase (CAL B, Novozym 435) regioselectively afforded 32-monoacetate $^5$, as a unique product, with a percent conversion depending on the employed solvent (Table 1) (Scheme 1).

This result, on the other hand, in agreement with the reported$^8$ selective lipase-catalyzed 32-acylation of tacrolimus type macro-lides,$^9$ was not suitable for our purposes. However, the very high CAL B regioselectivity toward position 32 suggested us to employ the same enzyme but with a different approach. Starting from 24,32-di-acetate $^6$, easily prepared from ascomycin $^2$ by treatment with acetic anhydride and dimethylaminopyridine (DMAP) in pyridine,$^{10}$ the selective removal of 32-acetate, by means of a CAL B-catalyzed alcoholysis, with tert-butyl methyl ether (TBME) as solvent and $n$-octanol as acyl acceptor, was observed (Scheme 1). Best conditions of alcoholysis, in order to obtain 24-monoacetate $^7$, $^{11}$ were found after a screening of solvents and acyl acceptors as reported in Table 2.

Evidences about enzymatic reaction outcome were preliminarily obtained by means of a $^1$H NMR analyses comparison with the literature data of ascomycin $^2$$^{12}$ and 24,32-di-O-formyl-ascomycin $^8$$^3$. In fact $^1$H NMR data of acetates $^5$$^7$$^-$7 are not reported. Ascomycin $^2$ presents two signals, at 3.40 and 3.92 ppm, assigned to H-32 and H-24,$^{12}$ respectively. Whereas the former is overlapped to the OCH$_3$ signals the latter (i.e., H-24 signal) and the H-32 signal (at 4.71 ppm) of 24,32-diformate $^5$ was more predictive for our purposes. The presence of a signal at 4.70 ppm, beside the signal at 3.92 ppm, allowed to conclude that the 32-monoacetate $^5$ was the unique product of CAL B-catalyzed transesterification. In a similar manner we assigned the structure of 24-monoacetate $^7$ to the product obtained from alcoholysis: the H-32 signal at 4.71 ppm was absent, indicating that the hydroxy group was not engaged in an ester bond; in addition, also the resonance due to the H-24 of 24-alcohol, at 3.92 ppm was absent whereas the region between 5.0 and 5.4 ppm, where usually the H-24 of esterified ascomycin (5.22 ppm for diformate $^5$) is present, was modified.

This preliminary assignment was later confirmed through a more complete NMR study as reported below.

Compound $^7$ was treated with polymer-bound triphenylphosphine (FLUKA) in carbon tetrachloride$^{13}$ to afford 32-chloro derivative $^9$ (40%); removal of 24-acetate was performed under acidic conditions (3 N HCl, 40% yields) to avoid the known degradations of ascomycin family macrocycles in presence of bases,$^{15}$$^{17}$ affording pimecrolimus $^1$ in only 14% overall yield (Scheme 2).

Since the presence of 24-acetate seemed to be crucial for the lowering of the yields of two final steps, we planned to prepare, through the same regioselective enzymatic approach, 24-silyl derivative $^4$, that is, the key intermediate of traditional syntheses of pimecrolimus $^1$ (Scheme 3).

Starting from the previously enzymatically obtained 32-monoacetate $^5$, by silylation with TBDMSOTf (5 equiv), in presence of 2,6-lutidine, 24-O-TBDMSS-32-O-acetyl derivative $^10$ was recovered; removal of 32-acetyl group was achieved by means of a CAL B-catalyzed alcoholysis,$^{18}$ affording intermediate $^4$ in good yields (80% from $^5$). By these mild conditions, typical of enzyme-catalyzed transformations, problems related to acidic or basic treatments of ascomycin family compounds were avoided.

![Scheme 1](image1.png)

**Scheme 1.** Regioselective Candida antarctica lipase B-catalyzed transformations of ascomycin $^2$ and its diacetate $^6$.

![Scheme 2](image2.png)

**Scheme 2.** CALB-catalyzed hydrolysis or alcoholysis of $^6$ to $^7$.

![Scheme 3](image3.png)

**Scheme 3.** CALB-catalyzed alcoholysis of $^4$ to $^5$.

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**Table 1**

<table>
<thead>
<tr>
<th>Lipase</th>
<th>Solvent</th>
<th>Time (h)</th>
<th>Conversion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PFL</td>
<td>Chloroform</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>PPL</td>
<td>Toluene</td>
<td>94</td>
<td>0</td>
</tr>
<tr>
<td>CCL</td>
<td>Toluene</td>
<td>92</td>
<td>0</td>
</tr>
<tr>
<td>CAL B</td>
<td>Acetonitrile</td>
<td>53</td>
<td>30</td>
</tr>
<tr>
<td>CAL B</td>
<td>tert-Butyl methyl ether</td>
<td>53</td>
<td>10</td>
</tr>
<tr>
<td>CAL B</td>
<td>Toluene</td>
<td>80</td>
<td>100</td>
</tr>
</tbody>
</table>

$^a$ From TLC and $^1$H NMR.

**Table 2**

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Acyl acceptor</th>
<th>Time (h)</th>
<th>Conversion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toluene</td>
<td>H$_2$O</td>
<td>80</td>
<td>0</td>
</tr>
<tr>
<td>Toluene</td>
<td>Methanol</td>
<td>48</td>
<td>0</td>
</tr>
<tr>
<td>Toluene</td>
<td>Ethanol</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Toluene</td>
<td>$n$-Butanol</td>
<td>120</td>
<td>30</td>
</tr>
<tr>
<td>tert-Butyl methyl ether</td>
<td>$n$-Octanol</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

$^a$ From TLC and $^1$H NMR.
biocatalyzed alcoholysis step it is possible to recycle the same enzyme sample used in the previous 32-acylation step, without significant loss of activity; in fact, if fresh CAL B is used, a comparable time is required to achieve the same conversion percent of 10 to 4 (about quantitative). Again, in order to introduce the chlorine at position 32, polymer bound triphenyl phosphine was used, leading to 11. We observed that when, after a 60–70% transformation, the polymer is removed and replaced with fresh reagent a nearly complete conversion is achieved and the 32-chloro derivative 11 can be used in the next step without any further purification. PTSA in dichloromethane/methanol (1/1) at 25–30 °C, among the tested conditions (hydrochloric acid in methanol, PTSA in tetrahydrofuran/water) gave best results for 24-hydroxy group deprotection; pimecrolimus 1 was recovered in 46% yield, after separation, by column chromatography, from a less polar compound (30–35%), identified as 23-dehydropimecrolimus.

Scheme 2. Final steps of the synthesis of pimecrolimus 1 from monoacetate 7.

Scheme 3. Chemoenzymatic synthesis of pimecrolimus 1 from monoacetate 5.
12, 23-24 The presence of this compound is common to other deproto-
ction methods, for example, hydrofluoric acid in acetonitrile,4 in
variable elevated amounts.

Overall yields (29%) of pimecrolimus 1 from ascomycin 2, were
comparable to these observed when we prepared intermediate 4
through the reported bissilylation–monodesilylation process.4

2.2. NMR study of acetates 5–7

To ascertain the structure of 5–7 a more accurate NMR study25
was done and, through 1D and 2D COSY, HSQC, and HMBC exper-
iments, it was possible to unambiguously establish the acetyl posi-
tions in these compounds. The experiments were carried out in
Pyd4, at 323 K. In fact using these experimental conditions the spec-
tra showed a good spread of the proton resonances and, especially,
of all the methyl groups. Two sets of signals are identified, since the
macrolactam system exists as a mixture of two rotamers in a 60/40
ratio, as in the case of ascomycin.2, 26 The study started with 24,32-
diacetate 6 and was focused on the signals of ascomycin moiety
carrying the acetyl groups. As an entry point for the study of
variable elevated amounts.

5. HMBC cross peaks between H-32 and a carbonyl at 169.7 ppm,
to be linked to a carbon carrying an acetyl group from the presence
by means of HSQC and HMBC, respectively. Also H-32 was shown
were shown to be correlated to the same carbon (C-31, 80.5 ppm)
ments. In particular, the resonances assigned to H-31 and to a
(2.42 ppm, major and minor rotamers), H-30a (1.20, major and
and 5.51 ppm, major and minor rotamers) because of an allylic
From H-26 it was also possible to assign, by COSY, H-28 (5.40
chlorosilane.13 Key intermediate 4 was obtained through two regiose-
lective lipase-catalyzed steps, can be useful not only for the
preparation of pimecrolimus but also for the preparation of other
32-substituted derivatives of ascomycin.21, 27

In order to unambiguously ascertain the outcome of lipase-cata-
lyzed transformations a careful high field NMR study, through 1D
and 2D experiments, was performed. This investigation has led to
the complete assignation of proton resonances of both rotamers of
monoaacetates 5.7 and diacetate 7, in the moiety directly involved
in the enzymatic transesterification and alcoholysis.

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cardo Monti for technical assistance and Professor Fiamma Ronchetti for helpful discussions.

References and notes

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493–503; (c) Stuetz, A.; Baumann, K.; Grassberger, M.; Wolf, K.; Meingassner,


3. Conclusion

Through a chemoenzymatic approach the synthesis of pime-
crolimus 1, from ascomycin 2, was realized with satisfactory yields,
considering the very high value of the final product; moreover dur-
ning the regioselective CAL B-catalyzed steps, performed in mild
conditions, only the desired products are obtained in absence of
rearrangements or degradation processes very frequently observed
in the case of sensitive molecules as ascomycin and related com-
ponents, containing a large number of functionalities.5, 15–17 The
same sample of immobilized CAL B can be easily recycled for either
transesterification or alcoholysis, without loss of activity. Use of
polymer-bound triphenyl phosphine, for the introduction of
chlorine at position 32, in addition to high yields, allows a simple
recovery of reagent that can be regenerated by treating with tri-
chlorosilane.13 Key intermediate 4, obtained through two regiose-

3484.245, 2935.287, 1735.331, 1649.741, 1450.039, 1372.278 cm

3491.528, 2935.860, 1744.728, 1710.227, 1652.310, 1448.662, 1371.335 cm

856.4 [M+Na]+.

898.4 [M+Na]+.

1874.728, 1735.331, 1649.741, 1450.039, 1372.278 cm

464, 895, A2, 1992. The reaction progress was monitored by TLC (hexane/acetone
65/35). After stirring (CAL B, Novozym 435, FLUKA, 0.140 g, 2 U/mg) were added. The
antarctica

10. Tacrolimus is the 21-allyl analogue of ascomycin.

11. T. Ronchetti for helpful discussions.

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18. 24-O-tert-Butyldimethylsilyl, 32-O-acetyl-ascomycin 10. DSC endothermic peak of fusion 236.43 °C; \(\Delta H^\circ = -81.4 \text{ (c 0.5 CHCl}_3\); 1H NMR (CDCl\textsubscript{3}) selected data of major rotamer (ppm) 0.05 (s, CH\textsubscript{3}Si), 0.06 (s, CH\textsubscript{3}Si), 0.89 (s, (CH\textsubscript{3})\textsubscript{3}C), 2.10 (s, CH\textsubscript{2}CO), 4.08 (m, H-24), 4.71 (m, H-32). IR \(\nu_{\text{max}}\) 3468.61, 2937.83, 1738.37, 1690.26, 1634.72, 1445.63 cm\(^{-1}\). MS (ESI\textsuperscript{+}) \(m/z\) 832.5 [M+Na\textsuperscript{+}]. 1H and \(^{13}\text{C} NMR data were in agreement with those reported in literature (Ref. 5).
19. The same transformation was successfully (73%) realized also with the same conditions utilized for preparation of 24-O-acetyl-ascomycin 7 from 24,32-diacetate 6. Chemical-physical data of 4 are in agreement with those reported in literature (Ref. 5).
20. The same transformation was successfully (73%) realized also with PTSA in benzene, at reflux, is reported to be applicable, on the contrary, in the case of 24-monoacetate dichlorotriphenylphosphorane (Grassberger, M.; Horvath, A. WO 040111 A2, 2006) not applicable, on the contrary, in the case of 24,32-disilyl derivative. In this case, we observed that lower temperatures (10–15 °C instead of 25–30 °C) and shorter times (20 h instead of 72 h) were required.
21. PTSA is reported (Ok. H. O.; Szumiloski, J. L.; Beattie, T. R.; Goulet, M. T. Bioorg. Med. Chem. Lett. 1997, 7, 2199–2204) to selectively remove the 32-silyl group from 24,32-diaceym 11. Selected NMR chemical shifts, as \(\delta\) (ppm) relative to residual pyridine fixed at 7.19 (higher field signal) for 1H NMR spectra and relative to Pyd\textsubscript{5} fixed at 123.0 ppm (higher field signal, central line) for \(^{13}\text{C} NMR spectra.
22. Pimecrolimus 1. To a solution of compound 11 (1.23 g, 1.35 mmol) in dichloromethane/methanol (1/1, 11 mL) PTSA was added (0.100 g, 0.53 mmol). The mixture was kept, under stirring, at 20–25 °C for 72 h, monitoring the reaction progress by TLC (hexane/acetone 8/2). A sodium hydrogen carbonate (0.04 g) aqueous solution (6 mL) was added; the organic phase was washed with brine and water, dried over sodium sulfate. After solvent evaporation at reduced pressure crude pimecrolimus, as a foam, was recovered. Silica gel column chromatography (hexane/acetone 8/2) afforded pure pimecrolimus that was crystallized from ethyl acetate/cyclohexane/water (0.5 g, 46%). DSC experiments (standard BRUKER pulse program). Chemical shifts are reported as \(\delta\) (ppm) of 5 and 7 (major and minor rotamer resonances are reported in the order). Compound 5: 25-CH\textsubscript{3} doublet (1.20 and 1.18), H-25 (2.10 and 2.24), H-24 (4.47 and 4.55), H-26 (5.78 and 5.81), H-28 (5.43 and 5.42), H-29 (2.40, major and minor rotamers), H-30a (1.18, major and minor rotamers), H-30b (2.15, major and minor rotamers), H-31 (3.33, major and minor rotamers), C-31 (80.4), 31-OCH\textsubscript{3} singlet (3.36 and 3.35), H-32 (4.94, major and minor rotamers), CO (32-acetyl) (169.8), CH\textsubscript{3} (32-acetyl) singlet (2.03 and 2.02). Compound 7: 25-CH\textsubscript{3} doublet (1.13 and 1.11), H-25 (2.19 and 2.42), H-24 (5.50 and 5.65), CO (24-acetyl) (169.5), CH\textsubscript{3} (24-acetyl) singlet (2.05 and 2.07), H-26 (5.48 and 5.52), H-28 (5.44 and 5.45), H-29 (2.44, major and minor rotamers), H-30a (1.18, major and minor rotamers), H-30b (2.15, major and minor rotamers), C-31 (80.7), 31-OCH\textsubscript{3} singlet (3.46 and 3.44), H-32 (3.68, major and minor rotamers).

24. The presence of a multiplet at 4.59–4.61 ppm (H-2 and H-32) and two double doublets at 6.21 (\(J = 15.7\text{ Hz}, J = 1.7\text{ Hz}, H-23\)) and 6.86 (\(J = 15.7\text{ Hz}, J = 5.3\text{ Hz}, H-24\)) ppm, respectively, in \(\text{H}^1\) NMR spectrum, allowed to assign to the main side product of deprotection step the structure of \(\Delta^3\)-pimecrolimus 12. An additional confirmation derived from MS (ESI\textsuperscript{+}) spectrum for the presence of a peak at 814.4 m/z [M+Na\textsuperscript{+}]; pimecrolimus (exact mass 809.4) spectrum shows the main peak at 832.4 m/z.
25. \(\text{H}^1\) NMR analysis was performed at 500 MHz with a Bruker FT-NMR AVANCE\textsuperscript{D} DRX500 spectrometer using a 5 mm z-PFG (pulsed field gradient) broadband reverse probe, and \(^{13}\text{C} NMR spectra were collected at 125.76 MHz at 323 K. The signals were unambiguously assigned by 2D COSY HSQC and HMBC experiments (standard BRUKER pulse program). Selected NMR chemical shifts are reported as \(\delta\) (ppm) of 5 and 7 (major and minor rotamer resonances are reported in the order). Compound 5: 25-CH\textsubscript{3} doublet (1.20 and 1.18), H-25 (2.10 and 2.24), H-24 (4.47 and 4.55), H-26 (5.78 and 5.81), H-28 (5.43 and 5.42), H-29 (2.40, major and minor rotamers), H-30a (1.18, major and minor rotamers), H-30b (2.15, major and minor rotamers), H-31 (3.33, major and minor rotamers), C-31 (80.4), 31-OCH\textsubscript{3} singlet (3.36 and 3.35), H-32 (4.94, major and minor rotamers), CO (32-acetyl) (169.8), CH\textsubscript{3} (32-acetyl) singlet (2.03 and 2.02). Compound 7: 25-CH\textsubscript{3} doublet (1.13 and 1.11), H-25 (2.19 and 2.42), H-24 (5.50 and 5.65), CO (24-acetyl) (169.5), CH\textsubscript{3} (24-acetyl) singlet (2.05 and 2.07), H-26 (5.48 and 5.52), H-28 (5.44 and 5.45), H-29 (2.44, major and minor rotamers), H-30a (1.18, major and minor rotamers), H-30b (2.15, major and minor rotamers), C-31 (80.7), 31-OCH\textsubscript{3} singlet (3.46 and 3.44), H-32 (3.68, major and minor rotamers).