

TITLE**“METHOD FOR PREPARING A SALT OF ISOCYCLOSPORIN A”****FIELD OF THE INVENTION**

The present invention belongs to the technical field of drug synthesis. In particular, the
5 present invention is related to a method for preparing a salt of isocyclosporin A, in
particular by transesterification of cyclosporin A into a salt of isocyclosporin A.

BACKGROUND ART

Cyclosporines are oligopeptides with a cyclic structure with antifungal and
immunosuppressive properties, used to modulate the body's immune response in organ
10 transplantations, to prevent rejection.

Since the original discovery of cyclosporin, several natural cyclosporines have been
isolated and identified, whereas non-natural cyclosporines have been obtained by
semisynthetic methods or through the application of culture techniques. Cyclosporin A
is the cyclosporin mostly used as drug.

15 The main indication of cyclosporin A, used in monotherapy or in association with other
immunosuppressive drugs, is the prevention of rejection in organ transplantation, in
particular in kidney, pancreas, liver and heart transplantations.

Cyclosporin A can also be used for the treatment of autoimmune diseases such as for
example uveitis, rheumatoid arthritis, psoriasis and ulcerative colitis.

20 Cyclosporin has a complex chemical structure, as it is formed by 11 peptides and
contains several N-methylated amino acids. As a result, the synthesis by peptide
condensation reagents is rather time-consuming and complicated. Therefore, at
present, the method mostly used for the synthesis of cyclosporin is by fermentation of
two fungi: *Trichoderma polysporum* and *Cylindrocarpon lucidum* (Survase, S. A.,
25 Kagliwal, L. D., Annapure, U. S. & Singhal, R. S. *Cyclosporin A - a review on
fermentative production, downstream processing and pharmacological applications*.
Biotech. Advances. 29, 418–435 (2011). However, this method of synthesis does not
allow a high yield of cyclosporin.

In 2010, the research group led by the chemist Danishevsky tried to synthesize
30 cyclosporin A by condensation reaction of isonitrile in the liquid phase. However, this
method of synthesis requires the use of many condensation reagents and is, therefore,

complicated. At present, therefore, the solid-phase synthesis cannot be realized, except with many difficulties and over long periods of time.

Furthermore, the use of cyclosporin A is limited by its low bioavailability and high toxicity, in particular nephrotoxicity. In fact, after the oral administration of cyclosporines, the concentration level in the blood reaches a high peak, followed by a rapid decline. Consequently, the oral administration of effective quantities of cyclosporin can lead to transient but dangerously high concentrations of cyclosporin in the blood at the peak level of blood concentration, resulting in several side effects, in particular kidney and liver damages.

It has been recently observed that some isocyclosporines, in particular isocyclosporines A, B, D and G, have an improved pharmacokinetic profile with respect to cyclosporines. Advantageously, the isocyclosporines, i.e. the isomers of cyclosporines, are absorbed by the intestine in the relatively inactive and non-toxic iso-form and are subsequently converted into the pharmacologically active cyclosporin form, thus reducing peak concentrations in the blood after the administration.

The purpose of the present invention is to provide a method for preparing a salt of isocyclosporin A in order to overcome the issues encountered in cyclosporin synthesis methods present today, briefly described above.

SUMMARY OF THE INVENTION

The Applicant has developed a method for preparing a salt of isocyclosporin A by direct conversion of cyclosporin A. The isocyclosporin obtained by the method according to the invention can be used as drug instead of cyclosporin, because the isocyclosporin has a better pharmacokinetic profile.

A first embodiment of the present invention refers to a method for preparing a salt of isocyclosporin A by transesterification of cyclosporin A into a salt of isocyclosporin A, which comprises the steps of:

- a) dissolving said cyclosporin A in anhydrous methanol and adding trifluoroacetic acid;
- b) heating the solution obtained according to step a) to a temperature ranging from 50°C to the reflux temperature of the reaction mixture for a time ranging from 30 to 60 hours;

c) removing said methanol and excess of said trifluoroacetic acid;

d) recovering the salt of isocyclosporin A with said trifluoroacetic acid,

wherein the molar ratio of said trifluoroacetic acid and said methanol in the solution obtained according to step a) is 1:3.

5 The Applicant has observed that with the optimal molar ratio between acid compound, in particular trifluoroacetic acid, and methanol of 1:3 a conversion of 53% of cyclosporin A in isocyclosporin A without having by-products is obtained, whereas with molar ratios between acid compound and methanol of 1:1 or 1:4 there is a lower conversion (30% or 20%) of cyclosporin A in isocyclosporin A (see examples 1 and 2). On the other hand,
10 with a molar ratio between acid compound and methanol of 3:1 there is a greater conversion of cyclosporin A in isocyclosporin A (75%) but with a higher number of by-products (example 2).

In light of this, the molar ratio of 1:3 resulted to be the best ratio because the 53% of cyclosporin is converted in iso-form, without by-products, so that the unconverted
15 residue can be recycled.

In a second embodiment of the present invention, the solution of cyclosporin A and methanol according to step a) is heated by microwave.

According to said second embodiment, the method for preparing a salt of isocyclosporin A by transesterification of cyclosporin A into a salt of isocyclosporin A comprises the
20 steps of:

a) dissolving said cyclosporin A in anhydrous methanol and adding trifluoroacetic acid;

b) heating in a microwave oven the solution obtained according to step a);

c) removing said methanol and the excess of said trifluoroacetic acid;

25 d) recovering the salt of isocyclosporin A with said trifluoroacetic acid.

In particular, microwave heating according to step b) of the method is carried out at a temperature ranging from 55 °C to 65°C for a time ranging from 10 to 20 hours, preferably of about 15 hours.

In a particularly preferred embodiment, step b) is carried out at 60°C for 15 hours.

30 In fact, microwave heating at 60 °C for 15 hours allows to obtain a yield of isocyclosporin A or of a salt thereof of 100%.

A second aspect of the second embodiment of the present invention refers to a continuous flow microwave system for preparing a salt of isocyclosporin A according to the method of the present invention.

Said continuous flow microwave system comprises one or more dispensing units of starting reagents, one or more microwave reactors and one or more product collectors.

5 In particular, the starting reagents are supplied in one or more microwave reactors using one or more pumps, preferably one or more HPLC pumps or syringe pumps.

The system according to the invention has also one or more coolers, and one or more back pressure regulators.

10 In a preferred embodiment, said continuous flow microwave system comprises multiple microwave reactors in parallel.

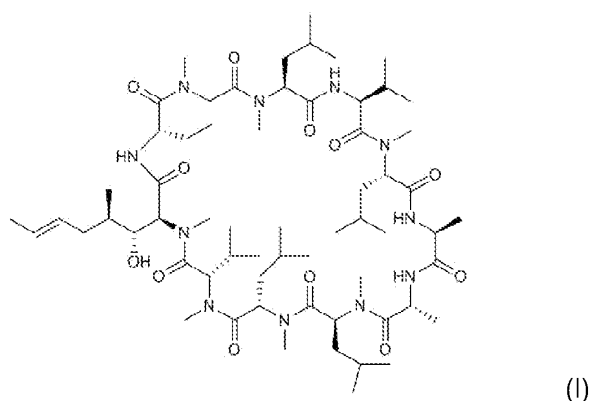
The combination of microwave heating with the continuous flow technique advantageously allows to increase the yields of isocyclosporin A obtained.

DETAILED DESCRIPTION OF THE INVENTION

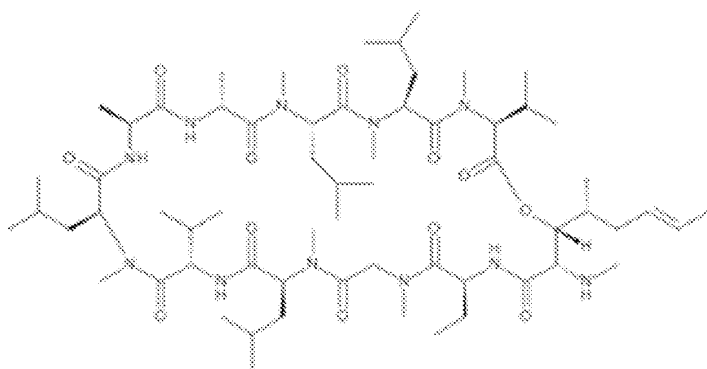
15 In order to reduce the side effects due to high concentrations of cyclosporin in the blood after the oral administration, the Applicant has conceived a method for preparing a salt of isocyclosporin A, isomer of cyclosporin A, which provides for the transesterification of cyclosporin A into a salt of isocyclosporin. This method allows to overcome the problems encountered in the methods for preparing a salt of isocyclosporin A, due to its

20 complicated chemical structure.

In fact, cyclosporin is a hydrophobic cyclical undecapeptide having the following formula I:

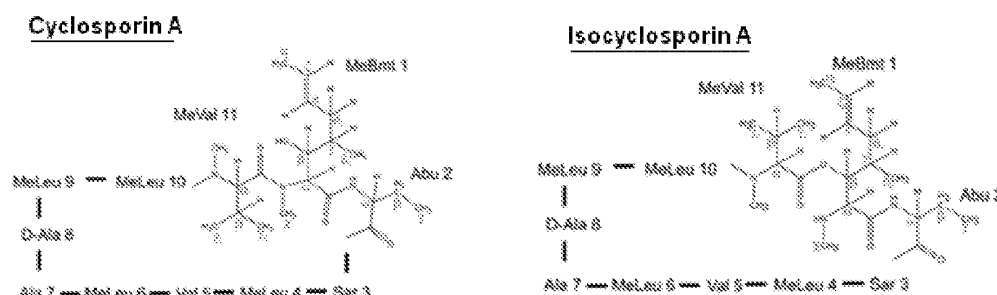


The isocyclosporin A, isomer of the cyclosporin A has instead the following formula (II) :



(II)

The structural differences between the cyclosporin A and its isomer are represented in the following scheme 1:



Scheme 1

Isocyclosporin A is absorbed by the intestine in the iso-form, which is relatively inactive and non-toxic, and subsequently converted into the pharmacologically active form of cyclosporin, thus reducing peak concentrations in the blood after the administration. Therefore, isocyclosporin A can be used instead of cyclosporin A as it has the same pharmacological effects but is less toxic.

Object of the first embodiment of the present invention is a method for preparing a salt of isocyclosporin A by transesterification of cyclosporin A into a salt of isocyclosporin A, which comprises the steps of:

- a) dissolving said cyclosporin A in anhydrous methanol and adding trifluoroacetic acid;

b) heating the solution obtained according to step a) to a temperature ranging from 50°C to the reflux temperature of the reaction mixture for a time ranging from 30 to 60 hours;

c) removing said methanol and the excess of said trifluoroacetic acid;

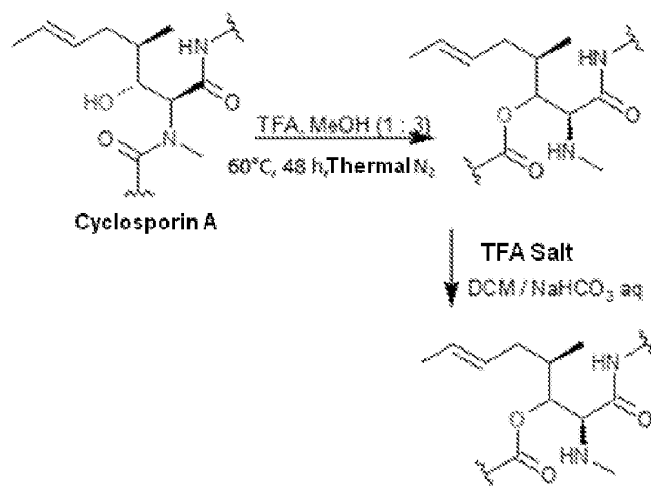
5 d) recovering the salt of isocyclosporin A with said trifluoroacetic acid, wherein the molar ratio of said trifluoroacetic acid and said methanol in the solution obtained according to step a) is 1:3.

The Applicant has advantageously observed that a molar ratio between trifluoroacetic acid and methanol in the solution formed in step a) (comprising cyclosporin A, trifluoroacetic acid and methanol) equal to 1:3 allows to obtain a yield of salt of isocyclosporin A of 80%. In an embodiment, the solution according to step a) comprises about 2 mmol of cyclosporin A and 60 mmol of methanol (see table 1 in example 1).

In particular, in the method according to the invention, step b) is carried out at a temperature ranging from 50°C to the reflux temperature of the reaction mixture, preferably at the temperature of 60°C.

In a particularly preferred embodiment, the solution according to step a), i.e. comprising cyclosporin A dissolved in methanol, is heated for 48 hours, preferably at the temperature of 60°C.

The reaction scheme according to the method of the invention is indicated below:



Scheme 2

The excess of trifluoroacetic acid in step c) can be removed by stripping with diethyl ether under vacuum.

As it is possible to observe in the above indicated scheme 2, to the obtained salt of isocyclosporin A with trifluoroacetic acid DCM/ NaHCO_3 can be added to remove the starting cyclosporin during the salification step (step d').

After the salt of isocyclosporin A with trifluoroacetic acid is recovered, the method eventually comprises dissolving the salt of isocyclosporin A with trifluoroacetic acid obtained in step d) in a solution comprising an acid compound selected from citric acid and lactic acid and methanol.

In particular, the method according to the invention may comprise downstream of step d) the following steps:

e) dissolving an acid compound selected from citric acid and lactic acid in methanol;

f) dissolving said salt of isocyclosporin A with said trifluoroacetic acid in the solution obtained in step e) while stirring the resulting solution for a time ranging from 0.5 to 2 hours;

g) removing the methanol and said trifluoroacetic acid; and

h) recovering the salt of isocyclosporin A with said acid compound selected from citric acid and lactic acid.

Examples of preparation of salt of isocyclosporin A with an acid compound selected from citric acid and lactic acid are indicated in the experimental section (examples 3 and 4).

The Applicant has observed that with the optimal molar ratio between trifluoroacetic acid and methanol of 1:3 a conversion of 53% of the cyclosporin A in isocyclosporin A without having by-products is obtained, whereas with molar ratios between acid compound and methanol of 1:1 or 1:4 there is a lower conversion (30% or 20%) of the cyclosporin A in isocyclosporin A (see examples 1 and 2).

With a molar ratio of acid compound to methanol of 3:1 there is a higher conversion of cyclosporin A in isocyclosporin A (75%) but with a higher number of by-products.

The total yield of isocyclosporin A obtained by the above-described method is 80%.

According to the second embodiment of the present invention, the method for preparing a salt of isocyclosporin A by transesterification of cyclosporin A into a salt of isocyclosporin A comprises the steps of:

- a) dissolving said cyclosporin A in anhydrous methanol and adding trifluoroacetic acid;
- b) heating in a microwave oven the solution obtained according to step a);
- c) removing said methanol and the excess of said trifluoroacetic acid; and
- d) recovering the salt of isocyclosporin A with said trifluoroacetic acid.

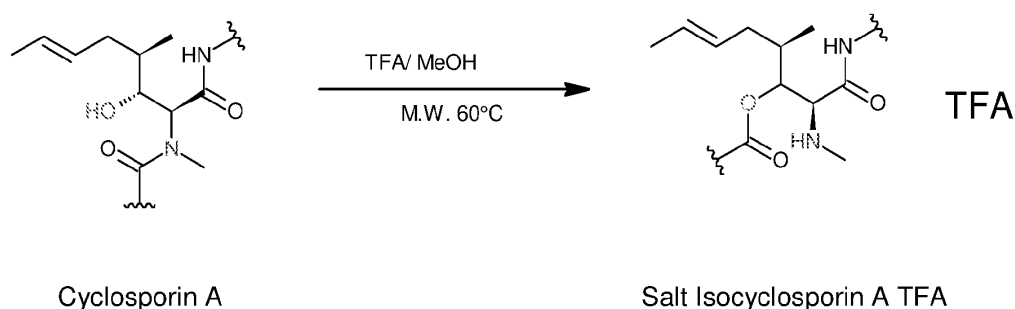
In particular, in step b) of the method the solution obtained according to step a) is heated in the microwave at a temperature ranging from 55 °C to 65°C, preferably at the temperature of 60°C.

In particular, step b) is carried out for a time ranging from 10 to 20 hours, preferably of about 15 hours.

In a particularly preferred embodiment, the microwave heating according to step b) of the method is carried out at 60 °C for 15 hours.

In fact, under these conditions it is possible to obtain a yield of a salt of isocyclosporin A of 100%.

Scheme 3 below shows the reaction scheme according to the method of the invention wherein the reaction solution between the compound (trifluoroacetic acid) and methanol is heated in the microwave (M.W.):



Scheme 3

In step c) of the method according to the invention, the excess acid compound is removed by stripping with diethyl ether under vacuum.

After the salt of isocyclosporin A with the trifluoroacetic acid is recovered (step d), the method comprises eventually dissolving said salt of isocyclosporin A with said trifluoroacetic acid in a solution comprising an acid compound selected from citric acid and lactic acid and methanol.

5 In particular, the method according to the invention may comprise downstream of step d) the following steps:

e) dissolving an acid compound selected from citric acid and lactic acid in methanol;

10 f) dissolving said salt of isocyclosporin A with said trifluoroacetic acid in the solution obtained in step e) while stirring the resulting solution for a time ranging from 0.5 to 2 hours;

g) removing the methanol and said trifluoroacetic acid; and

h) recovering the salt of isocyclosporin A with said acid compound selected from citric acid and lactic acid.

15 Examples of preparation of salt of isocyclosporin A with an acid compound selected from citric acid and lactic acid are indicated in the experimental section (examples 3 and 4).

The method for preparing a salt of isocyclosporin A comprising microwave heating can be carried out by a continuous flow system which comprises one or more microwave
20 reactors. In particular, the Applicant has observed that by combining the microwave heating with the continuous flow technique high yields of isocyclosporin A are obtained. Therefore, a second aspect of the second embodiment of the present invention refers to a continuous flow microwave system for preparing a salt of isocyclosporin A which comprises heating in the microwave oven the solution according to step a). In particular,
25 the continuous flow microwave system for preparing a salt of isocyclosporin A comprises one or more dispensing units of starting reagents, represented by the solution obtained in step a) of the method according to the present invention, one or more microwave reactors and one or more product collectors.

In particular, in said system the starting reagents are transported from the dispensing
30 units to microwave reactors by pumps, preferably HPLC pumps or syringe pumps.

The system may also comprise one or more coolers, and one or more back pressure regulators for monitoring the pressure.

Furthermore, there may also be sensors, as for example optical fiber sensors, for monitoring the reaction temperature.

- 5 In an embodiment, the system comprises a single dispensing unit of starting reagents, a single microwave reactor and a single product collector (as indicated in Figure 4). In this reactor, there is also one pump, one cooler and one back pressure cooler.

Preferably, the continuous flow microwave system comprises more than one dispensing unit of starting reagents, more than one microwave reactor and more than one product
10 collector. There may also be more than one pump, more than one cooler and more than one back pressure cooler.

More preferably, the continuous flow microwave system comprises multiple microwave reactors in parallel.

DESCRIPTION OF FIGURES

- 15 Figure 1 shows the liquid chromatography results of the salt of isocyclosporin A obtained by using a molar ratio of trifluoroacetic acid and methanol of 1:3. In Figure 1 "IsoCsA" indicates isocyclosporin A and "CsA" indicates cyclosporin A.

Figure 2 shows the liquid chromatography results of the salt of isocyclosporin A obtained by using a molar ratio of trifluoroacetic acid and methanol of 1:4 (see example 2). In

- 20 Figure 2 "IsoCsA" indicates isocyclosporin A and "CsA" indicates cyclosporin A.

Figure 3 shows the liquid chromatography results of the salt of isocyclosporin A obtained by carrying out the heating of the reaction mixture in the microwave. In Figure 3 "IsoCsA" indicates isocyclosporin A.

- Figure 4 is a graphical representation of the continuous flow microwave system
25 according to the invention. In particular, the continuous flow microwave system shown in Figure 4 comprises a dispensing unit (1), a microwave reactor (2) and a product collector (3). In Figure 4 are also shown a pump (4) that conveys the starting reagents from the dispensing unit (1) to the microwave reactor (2), a cooler (5) and a back pressure regulator (6).

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EXAMPLES

Example 1: Transesterification of cyclosporin A into a salt of isocyclosporin A with trifluoroacetic acid – molar ratio TFA: methanol of 1:3

The cyclosporin A (2.5 g, 2.08 mmol) has been dissolved in anhydrous methanol (2.45 ml). Trifluoroacetic acid (TFA) (1.5 ml) has been added and the reaction has been stirred at reflux at 60°C for 48 hours.

The solvent has been removed at reduced pressure and the excess of residual TFA has been removed by stripping with diethyl ether (2 x 15 ml) under vacuum.

The salt of Isocyclosporin A with dried TFA (1.33 g) looked like a white powder. A conversion of about 53% of the starting material and a quantitative yield of Iso-CsA have been obtained. In the final reaction no by-products were observed. The remaining starting material (cyclosporin A, CsA) has been removed during the step of salification by adding NaHCO_3 .

As it can be observed from Table 1, the molar ratio between trifluoroacetic acid and methanol is 1:3.

Table 1

Reagent	Molecular weight <i>gmol⁻¹</i>	Eq.	Moles <i>mmol</i>	Mass <i>g</i>	Volume <i>ml</i>	Density <i>gmL⁻¹</i>
Cyclosporin A (CsA) AK Scientific Lot 22C4I30I	1202.61	/	2.08	2.5	/	/
Trifluoroacetic acid (TFA)	114.02	/	20	/	1.5	1.489
Product Yields: Quantitative						
Salt isocyclosporin A with TFA (Iso-CsA · xTFA)	1316.62	/	1.11	1.33	/	/
<i>MeOH(3x mmol of TFA) = 60 mmol = 2.45 ml, d = 0.79 g/ml</i>						

The product has been characterized through liquid chromatography (see figure 1).

The Applicant has observed that by extending the reaction times beyond 60 hours, for example to 72 hours, there was no significant increase in the reaction yield, observing the formation of impurities. The increase of reaction times further than 60 hours was not considered to be advantageous.

Therefore, the Applicant has concluded that the optimal reaction conditions would be a molar ratio between trifluoroacetic acid and methanol of 1:3 for a reaction time to 60 hours.

Example 2: Transesterification of cyclosporin A into a salt of isocyclosporin A - molar ratio TFA: methanol of 1:1, 1:4, 3:1

The Applicant has carried out other experiments changing the molar ratio between trifluoroacetic acid and methanol according to what is indicated in Table 2.

Table 2

Cyclosporin A (CsA) 2.5gr (2.08 mmol) in salt Isocyclosporin A TFA						
Reagents	Molar Ratio (TFA/MeOH)	Time(h)	Mmol(TFA) mmol	% Conversion (CsA)	Volume MeOH ml	% Yield (IsoCsA)
a) Trifluoroacetic acid (TFA:MeOH)	1:1	> 48	20	30	2.45	50
b) Trifluoroacetic acid (TFA:MeOH)	1:4	> 48	20	20	9.8	35
c) Trifluoroacetic acid (TFA:MeOH)	3:1	48	60	75	0.85	/ (by-products)

As it can be observed from Table 2, with molar ratios between trifluoroacetic acid compound and methanol of 1:1 or 1:4 there is a lower conversion (30% or 20%) of cyclosporin A in isocyclosporin A. On the other hand, with a molar ratio between acid compound and methanol of 3:1 there is a greater conversion of cyclosporin A in isocyclosporin A (75%) but with a higher number of by-products.

The product has been characterized through liquid chromatography (see figure 2).

The isomerization yield obtained with molar ratios of trifluoroacetic acid and methanol of 3:1 (reagent c) in Table 2) has been measured after 12 hours and after 24 hours.

In particular, after 12 hours of reaction it has been observed a conversion yield of 55% and after 24 hours of 70%, without any appreciable improvement in the quantity of by-products detected.

The Applicant has furthermore observed that by operating at the same conditions indicated in Table 2 for the reagent c) but by increasing the reaction temperature from

60°C to 65°C, the conversion yield resulted to be of 77%, even in this case without any appreciable improvement in the quantity of by-products detected.

Example 3: Preparation of a salt of isocyclosporin A salt with citric acid

5 The salt of isocyclosporin A with trifluoroacetic acid (1 mmol) has been dissolved in a solution of MeOH with citric acid (1 mmol). The solution has been kept for 1 h under stirring, thereafter the solvent has been removed at reduced pressure and the excess of residual trifluoroacetic acid has been removed by stripping with diethyl ether (2 x 15 ml) under vacuum. 90% yield, of a salt of isocyclosporin A salt with citric acid.

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Example 4: Preparation of a salt of isocyclosporin A salt with lactic acid

The salt of isocyclosporin A with trifluoroacetic acid (1 mmol) has been dissolved in a solution of MeOH with lactic acid (1 mmol). The solution has been kept for 1 h under stirring, thereafter the solvent has been reduced at reduced pressure and the excess of residual trifluoroacetic acid has been removed by stripping with diethyl ether (2 x 15 ml) under vacuum. 91% yield, of a salt of isocyclosporin A salt with lactic acid.

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Example 5: Transesterification of cyclosporin A into a salt of isocyclosporin A – microwave heating

20 The cyclosporin A (2.5 g, 2.08 mmoles) has been dissolved in anhydrous methanol, then trifluoroacetic acid has been added (5 ml) and the reaction vial has been heated in the microwave at 60°C for 15 hours, by using the Biotage MW reactor.

The solvent has been reduced at reduced pressure and the excess of residual trifluoroacetic acid has been removed by stripping with diethyl ether (2 x 15 ml) under vacuum.

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The salt of Isocyclosporin A with dried TFA (3.386 g) looked like a white powder. The product has been characterized through liquid chromatography (see figure 3).

CLAIMS

1. Method for preparing a salt of isocyclosporin A by transesterification of cyclosporin A into a salt of isocyclosporin A, comprising the steps of:

- 5 a) dissolving said cyclosporin A in anhydrous methanol and adding trifluoroacetic acid;
- b) heating the solution obtained according to step (a) to a temperature ranging from 50°C to the reflux temperature of the reaction mixture for a time ranging from 30 to 60 hours;
- c) removing said methanol and the excess of said trifluoroacetic acid;
- 10 d) recovering the salt of isocyclosporin A with said trifluoroacetic acid,
- wherein the molar ratio of said trifluoroacetic acid and said methanol in the solution obtained according to step a) is 1:3.

15 2. Method according to claim 1, wherein step b) is carried out at a temperature of 60°C.

3. Method according to claim 1 or 2, wherein step b) is carried out for a time of about 48 hours.

20 4. Method according to any one of the preceding claims, wherein excess trifluoroacetic acid in step c) is removed by stripping with diethyl ether under vacuum.

5. Method according to any one of the preceding claims, further comprising downstream of step d) the following steps of:

- 25 e) dissolving an acid compound selected from citric acid and lactic acid in methanol;
- f) dissolving said salt of isocyclosporin A with said trifluoroacetic acid in the solution obtained in step e) while stirring the resulting solution for a time ranging from 0.5 to 2 hours;
- g) removing the methanol and said trifluoroacetic acid; and
- 30 h) recovering the salt of isocyclosporin A with said acid compound selected from citric acid and lactic acid.

6. Method according to any one of the preceding claims, wherein step a) comprises dissolving about 2 mmol of cyclosporin A in 60 mmol of methanol.

5 7. Method according to any one of the preceding claims, wherein the total yield of isocyclosporin A is 80%.

8. Method according to any one of the preceding claims, wherein the conversion percentage of the starting cyclosporin A is 53%.