Microbial Biotransformation Products of Cyclosporin A

MICHAELA KUHNT, FRANCIS BITSCH, JULIEN FRANCE, HANS HOFMANN,
JEAN-JACQUES SANGLIER and RENÉ TRABER*

Preclinical Research, Sandoz Pharma Ltd.,
CH-4002 Basle, Switzerland

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In order to mimic the human metabolic pathway of cyclosporin A (CyA) a total of 28 bacterial and 72 fungal strains was screened for their ability to transform CyA. Among 3 bacteria and 11 fungi, which produced the main human metabolite OL-17 [ηHyMeBmt^1]CyA, *Actinoplanes* sp. (ATCC 53771) achieved the best transformation rate (5.4%). Furthermore, the two *N*-demethylated minor products [Leu^4]CyA (3.2%) and [Leu^9]CyA (4.7%) were isolated, both known as minor natural metabolites and the first one also as a human biotransformation product. Microbial conversion of CyA using the actinomycete *Sebekia benihana* (NRRL 11111) yielded [γHyMeLeu^4]CyA (35%), [γHyLeu^4]CyA (4.5%) and [γHyMeLeu^4, γHyMeLeu^6]CyA (8.6%). The structures of these derivatives correspond with those of the human metabolic pathway. The related compounds [Nva^2]CyA (CyG) and [D-MeSer^3]CyA were similarly converted to the corresponding 4-γ-hydroxylated analogues. None of the biotransformation products showed a better immunosuppressive effect than CyA, although in various cases the cyclophilin binding affinity was comparable to that of CyA.

In the course of the human metabolic pathway, CyA is predominately hydroxylated at the terminal *C*-atom of amino acid 1 to the corresponding allyl alcohol [η-HyMeBmt^1]CyA (designated OL-17)[1]. Furthermore, hydroxylation takes place at the γ-position of the *N*-methylleucines 4, 6 and 9, as well as *N*-demethylation of the *N*-methylleucine 4[1]. The aim of the present study was to mimic the human metabolic pathway of CyA using the transformation talent of microorganisms. Microbial hydroxylation yielding regio- and stereoselective pure compounds in a one step reaction[2], represents an useful alternative to chemical synthesis. In the case of OL-17, for instance, chemical synthesis is a multi step reaction and yields an isomeric mixture[3], laborious to separate. The use of entire cells instead of isolated enzymes for hydroxylation renders the regeneration of costly cofactors redundant[4]. While in the field of steroids intensive research on stereoselective hydroxylation[5] has been performed, there are only a few reports on the biotransformation of macrocyclic molecules until now[6~8]. Therefore, a series of microorganisms with known hydroxylation capability was selected for a broad screening.

In order to investigate the type of biotransformation, HPLC-analysis using diode array detection was combined with LC-MS measurements. Derivatives, identified with these methods, were isolated out of larger scale fermentations, followed by the elucidation of their structures using NMR techniques.

The transformation products were tested for immunosuppressive activity, binding affinity to cyclophilin A and antifungal effects.

Materials and Methods

Microorganisms and Culture Conditions

Strains (72 fungi, 17 actinomycetes and 12 other bacteria) were purchased from the American Type Culture Collection (ATCC), the National Collection of Industrial and Marine Bacteria, the Northern Utilization Research and Development Division (NRRL) or the Centraalbureau voor Schimmelcultures (CBS). Strains were stored on agar slants at −25°C. Spores and cells of one agar culture were suspended in 10 ml 0.9% NaCl. 200-ml Erlenmeyer flasks each containing 50 ml seed culture medium (see below) were inoculated with 2 ml of these suspensions and incubated on a rotary shaker (200 rpm) at 24°C (fungi) or 27°C (bacteria) for 4 days. 100-ml Erlenmeyer flasks each containing 25 ml main culture medium (see below) were inoculated with 2.5 ml of the seed culture and incubated on a rotary shaker (200 rpm) at 24°C. After 24 hours CyA dissolved in MeOH was added to a final concentration of 100 mg/liter (screening) and 150 mg/liter (large scale fermentation) to the main culture. Cultures were harvested after 48, 72 and 96 hours of incubation.

Composition of the Liquid Seed Media

For bacteria: Medium 231 (screening/large scale fermentation): $CaCO_3$ 0.005/0.1%, glucose 0.7%, yeast extract (Gistex) 0.45%, malt extract (Wander) 0.5/1.0%, soluble starch 1.0%, N-Z-Amine Typ A (Sheffield)

Abbreviations: Bmt, (4*R*)-4-I(*E*)-2-butenyl]-4-methyl-L-threonine; Hy, hydroxy; Nva, L-norvaline.

0.25%, trace element soln.a 0.1% (v/v), pH 7.0. Trace element soln.a: H_3BO_3 0.01%, $FeSO_4 \cdot 7H_2O$ 0.5%, KI 0.005%, $CoCl_2 \cdot 6H_2O$ 0.2%, $CuSO_4 \cdot 5H_2O$ 0.02%, $MnCl_2 \cdot 4H_2O$ 0.2%, $ZnSO_4 \cdot 7H_2O$ 0.4%, H_2SO_4 (97%) 0.1% (v/v).

For fungi: Medium SA: Bacto agar 0.1%, yeast extract (Gistex) 0.4%, malt extract (Wander) 2.0%, pH 5.0~5.5. Medium SB: Soya protein (Siber & Hegner) 0.75%, dextrose (Difco) 2.0%, malt extract (Wander) 0.1%, brewer's yeast (Cenovis) 0.1%, KH_2PO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.005%, $CaCl_2 \cdot 2H_2O$ 0.002%, NaCl 0.001%, trace element soln.b 0.1% (v/v), Bacto agar 0.1%, pH 6.0~6.2. Trace element soln.b: $Na_2MoO_4 \cdot 2H_2O$ 0.003%, $ZnSO_4 \cdot 7H_2O$ 0.44%, $FeSO_4 \cdot 7H_2O$ 0.55%, $CuSO_4 \cdot 5H_2O$ 0.008%, $MnCl_2 \cdot 4H_2O$ 0.018%, H_2SO_4 (97%) 0.2% (v/v).

Composition of the Main Media

For bacteria: Medium Act 1: Glucose 0.5%, soluble starch 1.5%, N-Z-Amine Typ A (Sheffield) 1.0%, brewer's yeast (Cenovis) 0.2%, K_2HPO_4/KH_2PO_4 0.06/0.03%, $MgSO_4 \cdot 7H_2O$ 0.01%, $CaCl_2 \cdot 2H_2O$ 0.01%, NaCl 0.005%, trace element soln.a 0.1% (v/v), pH 6.2~6.5. Medium Act 3: Bacto Trypton 0.5%, Bacto yeast extract 0.3%, glucose 1.0%, pH 7.1. Medium Act 1.2 (large scale fermentation): Glucose 1.0%, dextrose (Difco) 1.0%, soluble starch 1.0%, yeast extract (Gistex) 0.25%, soyabean flour (Nurupan) 1.25%, K_2HPO_4/KH_2PO_4 0.025/0.012%, $MgSO_4 \cdot 7H_2O$ 0.01%, $CaCl_2 \cdot 6H_2O$ 0.005%, trace element soln.a 0.1% (v/v), pH 7.2~7.5.

For fungi: Medium MA: Glucose 2.0%, soya protein (Siber & Hegner) 0.2%, malt extract (Wander) 0.2%, yeast extract (Gistex) 0.2%, KH_2PO_4 0.2%, $MgSO_4 \cdot 7H_2O$ 0.05%, trace element soln.b 0.1% (v/v), Bacto agar 0.1%, pH 5.1~5.4. Medium MB: Soya protein (Siber & Hegner) 1.0%, dextrose (Difco) 3.0%, malt extract (Difco) 0.2%, brewer's yeast (Cenovis) 0.2%, KH_2PO_4 0.075%, $MgSO_4 \cdot 7H_2O$ 0.001%, $CaCl_2 \cdot 2H_2O$ 0.005%, NaCl 0.002%, trace element soln.b 0.15% (v/v), pH 6.0~6.2.

Extraction of the Screening Broths

20 ml ethyl acetate were added to 20 ml of the main culture, shaken for about 20 minutes at 24°C at 200 rpm and then centrifuged at 4500 rpm for 10 minutes. Approximately 15 ml of the ethyl acetate layer were evaporated to dryness and redissolved in 1.5 ml of MeOH. 10 μl of these extracts were then chromatographed on HPLC with a H_3PO_4 0.1%/CH_3CN gradient (30~100% CH_3CN in 14 minutes) on a RP-18 4 μm column at 75°C. Biotransformation products were detected at 210 nm (DAD).

LC-MS

Electrospray ionization mass spectrometry (ESI-MS) was performed on a Finnigan TSQ 7000 triple quadrupole mass spectrometer. The instrument was controlled and data were analyzed using ICIS software (Finnigan). The electrostatic-spray ion source was operated at 4.5 kV and atmosphere-vacuum transfer capillary was heated at 220°C. The column effluent was split 5:1 with a Valco tee allowing a flow rate of 0.05 ml/minute into the electrospray nebulizer. Full scan mass spectra were recorded from mass to charge ratio (*m/z*) 500 to 1400 in 1.8 seconds for the MS analysis of cyclosporins. The conditions for LC-MS were set up as followed: Column: Spherisorb 5, RP-18 (5 μm), gradient system: H_2O/CH_3CN 50~70% in 10 minutes, hold, column temperature: 75°C. UV-detection at 210 nm.

Isolation of Biotransformation Products of ATCC 53771

The culture broth (30 liters) was extracted with ethyl acetate. This extract was separated with MeOH on Sephadex LH-20 and fractions were monitored for **1b**, **1h** and **1i**, respectively, by analytical HPLC. Fractions containing one of these products were separated on silica gel (0.04~0.063 mm) using ethyl acetate saturated with water. Enriched fractions were purified by preparative HPLC under isocratic conditions (CH_3CN-H_2O-H_3PO_4 63:37:0.01) on a RP18 7 μm column at 210 nm and 75°C and yielded 64 mg **1b**, 57 mg **1h** and 10 mg **1i**, respectively.

Isolation of the Biotransformation Products of NRRL 11111

The mycelium was separated from the culture medium and the resulting culture filtrate (13 liters) was extracted three times with 1,2-dichloroethane using 1.5 liter at each extraction. The combined organic solutions were evaporated under vacuum at 40°C. The crude residue was subjected to Sephadex LH-20 gel filtration using methanol as eluent. Those fractions containing the cyclosporin derivatives (525 mg) were pooled and chromatographed on silica gel (0.04~0.063 mm, Merck) using $CHCl_3$/MeOH as eluent. Repeated chromatography using the same system yielded pure **1c** (110 mg) as an amorphous white powder, mp 150~153°C, $[\alpha]_D^{20}$ $-225°$ (*c* 0.53 in $CHCl_3$).

From large scale fermentations (6200 liter) the more polar side fractions obtained in the purification process were further separated by repeated silica gel chromatography (0.04~0.063 mm) using acetone - hexane 2:1 and *tert*.butylmethyl ether - MeOH - H_2O 90:9:1 as eluent, respectively. The first fraction contained **1d**, which was further purified by decolorizing with charcoal yielding the pure compound (6.0 g) as an amorphous white powder, mp 162~164°C, $[\alpha]_D^{20}$ $-211°$ (*c* 0.5 in $CHCl_3$). The later fractions from the above silica gel column chromatography yielded after being decolorized with charcoal 890 mg **1e** as an amorphous white powder, mp 157~160°C, $[\alpha]_D^{20}$ -217 (*c* 0.54 in $CHCl_3$).

Using cyclosporin G ([Nva^2]CyA) instead of CyA as starting material for the biotransformation with NRRL 11111, 230 mg **1f** was isolated from a 100 liter fermenta-

tion by repeated silica gel column chromatography using ethyl acetate saturated with water and acetone-hexane 2:1 as eluent, respectively. **1f** was obtained as an amorphous white powder, mp 138~141°C, $[\alpha]_D^{20}$ −213 (*c* 0.69 in $CHCl_3$). **1g**, mp 150~159°C, $[\alpha]_D^{20}$ −226 (*c* 0.75 in $CHCl_3$) was obtained from [D-MeSer[3]]CyA using analogous biotransformation and separation procedures.

^1H and ^{13}C NMR Spectroscopy

All spectra were recorded at 20°C on a Bruker AMX-400 spectrometer equiped with a 5 mm inverse triple resonance probe. The spectra were acquired in $CDCl_3$ or C_6D_6 using TMS as internal reference. Resonance assignments were obtained from a series of homonuclear and heteronuclear 2D experiments. Only the relevant signals are presented. The ROESY experiment was recorded with an in-house modification of the pulse sequence proposed by HWANG and SHAKA[9] (C. DALVIT, personal communication).

Biological Assays

MLR: The immunosuppressive activity was assessed with the mouse mixed lymphocyte reaction (MLR) by measuring the incorporation of labeled [^{3}H]thymidine into DNA[10] (all experiments were run at least in parallel).

Cyclophilin binding assay: Cyclophilin binding to human recombinant cyclophilin A was determined in a competitive ELISA test as described previously[11].

Antifungal effects: The antifungal spectrum of cyclosporin A is rather narrow and is limited, besides a few other organisms, to some strains of fungi imperfecti, *e.g. Curvularia lunata* and *Neurospora crassa*[12]. Growth inhibition becomes evident as deformation and branching of the hyphal tips, the so-called "ramification"[13]. All experiments were at least repeated three times and performed with 4 concentrations of metabolites dissolved in MeOH from 0.001 to 1 mg/ml on agar plates (yeast/malt extract medium) with the *Neurospora* strain Sandoz 261.

Results and Discussion

In order to find microbial transformed derivatives of CyA, comparable to those derivatives of the human metabolic pathway[1], 28 bacterial and 72 fungal strains, known for their hydroxylation potency or for their ability to transform molecules showing structure similarities with CyA, were selected for a broad screening. The actinomycete *Sebekia benihana* (NRRL 11111) attracted our attention because it achieved remarkable transformation rates (Table 1). As this strain is known to hydroxylate an allylic methyl group in novobiocin[14], one would expect the transformation of CyA **1a** to OL-17 **1b**.

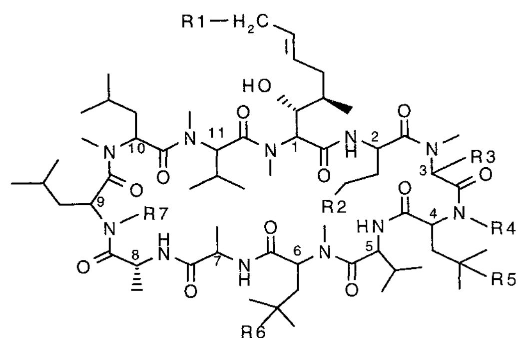
One of the derivatives **1c**, obtained by biotransformation of CyA with *Sebekia benihana*, turned out to be distinctly more polar than CyA. In the mass spectrum

Table 1. Strains converting cyclosporin A.

Compound	Relative retention α	Amount of derivative in %	Fungi	Bacteria
4-(γ-hydroxy)-CyA (**1c**)	4.68	2.0	*Emericella unguis* ATCC 10032	
		4.8	*Cunninghamella blakesleana* ATCC 49989	
		8.5	*Aspergillus malignus* CBS 16061	
		9.5		*Pseudomonas chlororaphis* ATCC 9447
		35.0		*Sebekia benihana* NRRL 11111
4-(γ-hydroxy)-4-*N*-demethyl-CyA (**1d**)	3.42	4.2	*Helicosphyllum puriforme* ATCC 423	
		2.4		*Brevibacterium healii* ATCC 15527
		4.5		*Sebekia benihana* NRRL 11111
Di-4,6-(γ-hydroxy)-CyA (**1e**)	3.12	8.6		*Sebekia benihana* NRRL 11111
OL-17 (**1b**)	3.45	Traces	*Aspergillus malignus* CBS 16061	
		0.01	*Thamnidium elegans* ATCC 8997	
		0.01	*Thamostylum piriforme* ATCC 8992	
		0.01	*Trichothecium roseum* ATCC 12519	
		0.05	*Botrytis eliptica* ATCC 11787	
		0.05	*Cunninghamella blakesleana* ATCC 49989	
		0.05	*Gliocladium deliquescens* ATCC 10097	
		0.06	*Chaetomium cuniculorum* ATCC 11201	
		0.08	*Penicillium chermesinum* ATCC 10424	
		0.19	*Penicillium notatum* ATCC 103	
		0.2	*Botrytis cinerea* ATCC 12481	
		0.1		*Pseudomonas chlororaphis* ATCC 9447
		0.86		*Streptomyces rimosus* ATCC 28893
		5.4		*Actinoplanes* sp. ATCC 53771
4-*N*-demethyl-CyA (**1h**)	5.69	Traces	*Cunninghamella blakesleana* ATCC 49989	
		3.2		*Actinoplanes* sp. ATCC 53771
9-*N*-demethyl-CyA(**1i**)	7.76	4.71		*Actinoplanes* sp. ATCC 53771
CyA (**1a**)	10			

Relative retention α (HPLC) = Rt (derivative) − Rt (mobile phase)/Rt (CyA) − Rt (mobile phase) × 10, Rt: Retention time. Amount of derivative in % determined by HPLC = AUC (derivative) × 100%/AUC (CyA in compound control), AUC: Area under curve.

Fig. 1. The structures of cyclosporin A, related compounds and conversion products.



Compound	R1	R2	R3	R4	R5	R6	R7
1a	H	H	H	CH_3	H	H	CH_3
1b	OH	H	H	CH_3	H	H	CH_3
1c	H	H	H	CH_3	OH	H	CH_3
1d	H	H	H	H	OH	H	CH_3
1e	H	H	H	CH_3	OH	OH	CH_3
1f	H	CH_3	H	CH_3	OH	H	CH_3
1g	H	H	CH_2OH	CH_3	OH	H	CH_3
1h	H	H	H	H	H	H	CH_3
1i	H	H	H	CH_3	H	H	H

the $[MH]^+$ value of m/z 1218 indicated that a mono hydroxylated product had been obtained. However, the fragment $[MH]^+-112$, which is typical for the elimination of $C_7H_{12}O$ from MeBmt, excluded that hydroxylation to OL-17 had taken place, because the expected fragment would have been $[MH]^+-128$. In the ^1H NMR spectrum of **1c** an additional signal at δ 3.8 ppm exchangeable with D_2O confirmed a new hydroxy group. This hydroxy group must be located in the γ-position of the amino acid 4 ($MeLeu^4$), because the signal of the α-proton of $MeLeu^4$ in **1c** was shifted to lower field (5.52 ppm) in respect to CyA (5.35 ppm) due to the influence of the neighbouring hydroxy function. Furthermore two new singlets at δ 1.22 and 1.27 ppm were associated with the methyl groups of $MeLeu^4$. One of the β-methylene protons of $MeLeu^4$ is shifted from 2.0 to 2.4 ppm; for the second one no chemical shift difference was measured. For the proton signals of all the other amino acids in **1c** no significant changes were observed in comparison to the ^1H NMR spectrum of CyA. In the ^{13}C NMR spectrum, a new singlet at δ 68.87 ppm confirmed the 4-γ-hydroxy substitution. On the basis of these data **1c** was identified as [$\gamma HyMeLeu^4$]-CyA. This derivative could not be detected in human urine, but a similar metabolite carrying an additional hydroxy group in γ-position of amino acid 9[1], was formed.

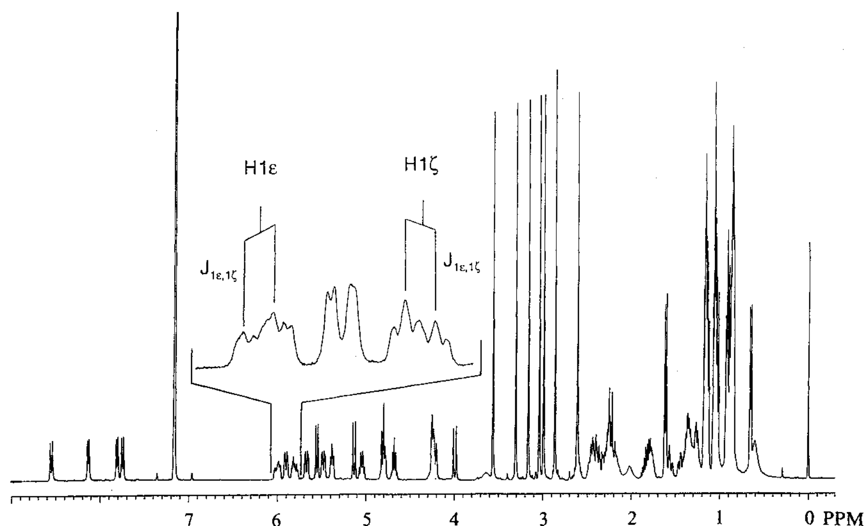
Further biotransformation products of CyA, obtained in minor quantities with NRRL 11111, were [$\gamma HyLeu^4$]-CyA **1d** and [$\gamma HyMeLeu^4$, $\gamma HyMeLeu^6$]CyA **1e**. The MH^+-values indicated for **1d** both N-demethylation and hydroxylation, and for **1e** dihydroxylation, respectively. A new doublet at δ 7.24 ppm in the ^1H NMR of **1d**, instead of the singulet at δ 3.1 ppm (4-N-CH_3) of **1a**, could be assigned to the new amide proton. Hydroxylation to **1d** was shown with a new signal at δ 71.2 ppm in the ^{13}C NMR spectrum. The fact, that in the amino acid analysis of **1d** no leucine could be detected, proofed that hydroxylation and N-demethylation had both taken place in the same unit $MeLeu^4$.

The biotransformation product **1e** turned out to be hydroxylated at $C\gamma$ of both $MeLeu^4$ and $MeLeu^6$. The structure elucidation was based on the interpretation of COSY, ROESY, HCCORR and HMBC spectra (4γ-C: 68.7 ppm, 6γ-C: 69.0 ppm). Both **1d** and **1e** differ from the human metabolites of CyA; the corresponding biotransformation products isolated from human urine are additionally hydroxylated in the γ-position of amino acid 9[1].

In order to study the influence of the substitution in the cyclosporin molecule on the specifity of the reactions using the strain NRRL 11111, experiments with structural analogues were performed. Modifications in the amino acids 2 ([Nva^2]CyA) or 3 ([D-$MeSer^3$]CyA) do not effect the position and rate of biotransformation. *Sebekia benihana* transformed these different cyclosporins to the corresponding 4-γ-hydroxy derivatives **1f** and **1g**, respectively. This reaction has been predominant for all tested cyclosporins so far, but side reactions, such as N-demethylation at $MeLeu^4$ in combination with the hydroxylation at $MeLeu^4$ to **1d**, can also be observed.

Although the main human metabolite OL-17 was not produced by *Sebekia benihana*, it could be detected as a minor transformation derivative of 11 fungi and 3 bacteria (Table 1). The actinomycete *Actinoplanes* sp. (ATCC 53771), actually known as a strain with demethylation capability[7], turned out to hydroxylate CyA to OL-17 best (5.4%), as determined by HPLC analysis. OL-17 was isolated from a 30 liter scale fermentation using the strain ATCC 53771. The regioselective hydroxylation at the terminal methyl group of the amino acid MeBmt was confirmed by ^1H NMR techniques. The DQF-COSY experiment and a modified version of the ROESY experiment (see Materials and Methods) were used to study the configuration of the double bond in the side chain of MeBmt of OL-17. A large coupling

Fig. 2. ^1H NMR spectrum of OL-17 (10 mg/0.5 ml C_6D_6).



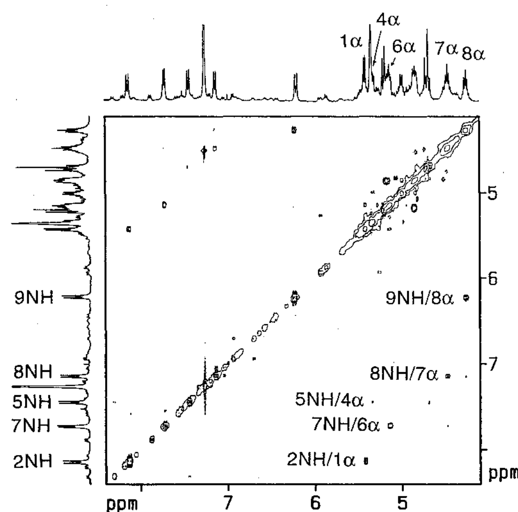
constant (15 Hz) between H1ε/H1ζ (Fig. 2) and a strong ROE between H1ε and H1η (data not shown) indicated a "*trans*" configuration for the olefinic protons. Therefore the configuration in MeBmt of OL-17 remained unchanged in respect to CyA and the structure corresponded to the main human metabolite of CyA[1].

In the course of the biotransformation of CyA with ATCC 53771 two further derivatives **1h** and **1i** were isolated. The mass spectra of both biotransformation products revealed a mass difference of −14 units compared to CyA, which indicated a demethylation. The spectroscopical data of **1h** were identical with these of [Leu⁴]CyA, a derivative available by chemical synthesis[15] and also recently found as a natural minor metabolite[16].

The ^1H NMR spectrum of **1i** showed a new doublet at δ 6.2 ppm, which was assigned to the 9-amide proton. This doublet replaced the singlet of the *N*-methyl protons at δ 3.1 ppm of MeLeu⁹ in the ^1H NMR spectrum of CyA. The *N*-demethylation at the amino acid 9 was further confirmed by COSY, ROESY (Fig. 3) and TOCSY experiments. Coincidently with our work, [Leu⁹]CyA was described as a new member of the cyclosporin group isolated from the fungus[17].

1b and **1h** are the only microbial biotransformation derivatives detected in the screening, which are identical to the corresponding human metabolites of CyA. Whereas the cytochrome P-450 mechanism resembles the chemical oxidation by hypervalent transition metal oxidants, the Flavine-dependent mechanism parallels the oxidation of organic compounds by peroxides[18].

Fig. 3. ROESY spectrum of [Leu⁹]CyA (10 mg/0.5 ml $CDCl_3$).



Cytochrome P-450-dependent monooxygenases are found in all organisms from bacteria to humans[19]. This fact may explain that *Sebekia benihana* and *Actinoplanes* sp. produce biotransformation products showing structural similarities to the human metabolites.

Biological Data

The *in vitro* profile of the hydroxylated and/or *N*-demethylated cyclosporin derivatives comprising immunosuppressive activity, binding affinity to cyclophilin A and antifungal effects is summarized in Table 2. The microbial hydroxylation and *N*-demethylation of CyA yielding different biotransformation products led

Table 2. Biological data of cyclosporin A biotransformation products.

Compound	MLR activity ratio	Cyclophilin binding ratio	Ramification assay
1a	1	1	3
1b	40	2	2
1c	>100	0.5	2
1d	>100	10	0
1e	>100	0.8	0
1f	>100	2.5	2
1g	>1000	0.16	0
1h	>25	40	2
1i	40	14	2

MLR: The mean IC_{50} for CyA was determined as 0.016 μg/ml. The values in Table 2 are expressed as the ratio IC_{50} of the test compounds to IC_{50} of CyA.

Cyclophilin binding affinity: The results represent relative values of binding affinity compared to CyA (IC_{50} for CyA 5.5×10^{-8} μg/ml). Values >1 mean lower affinity, values <1 mean higher affinity.

Antifungal effects: determined on the basis of a series of dilutions of each compound, measured in triplicates.

Graduation of the activity: 3; active up to 0.01 mg/ml, 2; active up to 0.1 mg/ml, 1; active by 1 mg/ml, 0; inactive.

to a significant decrease of the immunosuppressive effect compared to CyA. However, the cyclophilin binding affinity for the derivatives [γ-HyMeLeu^4]CyA (**1c**), [γ-HyMeLeu^4, γ-HyMeLeu^6]CyA (**1e**) and [D-MeSer^3, γHyMeLeu^4]CyA (**1g**) was slightly increased in respect to CyA. The reason for the reduced immunosuppressive activity lies in the modification of amino acid 4, which is part of the effector domain (amino acids 4→9), but not part of the cyclophilin binding domain (amino acids 10→3)[20]. The ramification assay is a model to detect either antifungal effects and differention of the hyphae of *Neurospora crassa* induced by metabolites[13]. (Ramihyphin A was later found to be identical to CyA[21]). None of the derivatives reached a ramification level higher than CyA. With a single hydroxylation of CyA in amino acid 1 or 4 or *N*-demethylation of amino acid 4 or 9 the ramification effect was reduced in respect to CyA, but remained at a significant level. Further modifications of the molecule led to a loss of antifungal activity.

Hydroxylation and *N*-demethylation are relevant detoxification reactions in the metabolic pathway of living beings[22]. This fact may be a possible reason for the reduced biological effects of the microbial transformation products in respect to CyA. On the other hand, the higher polarity of these derivatives may be advantageous for improved pharmacokinetic attributes. Furthermore, hydroxylated derivatives easier attainable by biotransformation than by chemical methods, may serve as starting materials for the synthesis of new products.

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