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Structure of Cyclosporine and Its Metabolites: Total Synthesis of Cyclosporine Metabolites Formed by Oxidation at Positions 4 and 9 of Cyclosporine. Preparation of Leucine-4-cyclosporine, (γ-Hydroxy)-*N*-methyl-leucine-9-cyclosporine and Leucine-4-(γ-hydroxy)-*N*-methyl-leucine-9-cyclosporine

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Abstract. The syntheses of the cyclosporine metabolites Leu-4-cyclosporine (AM4N), (γ-OH)MeLeu-9-cyclosporine (AM9) and Leu-4-(γ-OH)MeLeu-cyclosporine (AM4N9) were completed. Aspartic acid was used as starting material to prepare peptides containing (γ-OH)MeLeu residues. The structure of metabolite M13 was definitively established as being Leu-4-(γ-OH)MeLeu-9-cyclosporine. The immunosuppressive activity *in vitro* of these synthesized metabolites was compared with that of cyclosporine and metabolite AM1.

1. Introduction

1.1. *Introduction to Cyclosporine*

The immunosuppressive cyclic undecapeptide cyclosporine [1], the active constituent of Sandimmune (*Sandoz Pharma Ltd.*) has revolutionized the field of organ transplantation [2–4]. Its specific mechanism of action is the inhibition of lymphokines (*i.e.* interleukin-2) on the level of m-RNA-transcription upon T-lymphocyte activation [5]. For its *in vivo* and *in vitro* activities, the cytosolic binding protein cyclophilin has been proposed as its cellular receptor [6]. Now, two groups [7][8] have found that a complex of cyclosporine with cyclophilin interacts with another cytosolic protein – a phosphoprotein phosphotase named 'calcineurin' – known to associate with calmodulin, a Ca^{++}-binding protein, for activity. This suggested that cyclosporine could interfere with T-cell activation by altering the phosphorylation state of cellular proteins [8][9].

Preliminary results in our laboratories with cyclosporine derivatives suggest that the ability of cyclosporine-cyclophilin complexes to associate with calcineurin correlates with immunosuppressive potency [10]. Recently, it has been shown that non-immunosuppressive cyclosporine derivatives could tightly bind to cyclophilin and, thus, antagonize the immunosuppressive activity of cyclosporine *in vitro* [11]. In analogy to the immunosuppressant FK 506 (*Fugisawa Ltd.*) [12], a specific structure at the 'effector' domain of the cyclosporine molecule different from the cyclophilin ('immunophilin') 'binding' domain could determine the biological activity. All cyclosporine analogues showing significant immunosuppressive activity upon binding to cyclophilin interacted preferentially with the residues 1, 2, 10, and 11 of cyclosporine [13][14].

The structure of cyclosporine in single crystals and in apolar solvents has been determined and found to be similar (*Fig. 1A*) [15][16]. Today, the interaction between cyclophilin and cyclosporine in aqueous solution has been intensively studied by NMR techniques [17][18]. The structure of cyclosporine bound to cyclophilin represents a novel conformation of cyclosporine. The structure has all peptide bonds in the *trans* form and no intramolecular H-bonds, and exposes nearly all polar groups to its environment (*Fig. 1B* [17]). The binding domain of cyclosporine to its receptor established before [13][14] still correlates in both structures (*Fig. 1A* and *1B*), but now the MeBmt-1 residue is

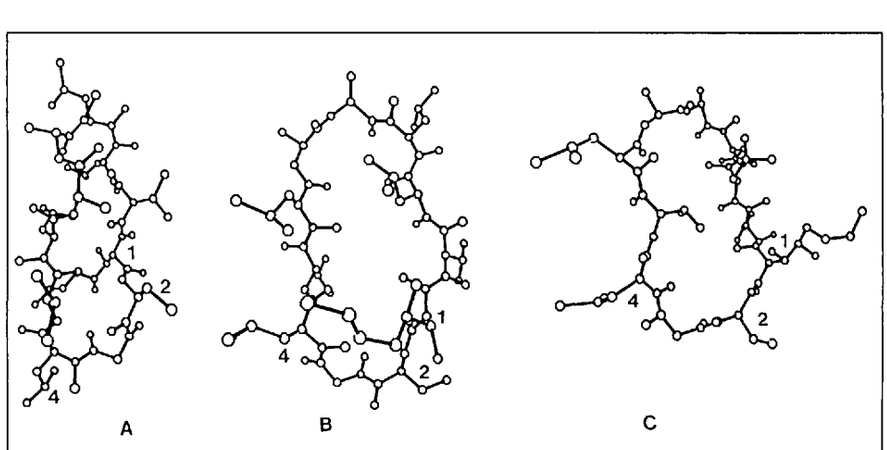

Fig. 1. *Conformations of cyclosporine,* A) *in crystal an in apolar solvents* [15][16]; B) *on cyclophilin* [17][18], *and* C) *on a monoclonal antibody* [19] (from Dr. *M. Walkinshaw*, Drug Design Group, *Sandoz Pharma Ltd.*)

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folded back onto the molecule on the upper face (*Fig. 1B*) rather than on the lower face (*Fig. 1A*) when keeping the same orientation of the cyclosporine amino acid residues as in *Fig. 1A* and *1B*.

A similar conformation to that found for cyclosporine on cyclophilin has been found for cyclosporine in an aqueous environment by the X-ray structure of a cyclosporine-Fab complex (*Fig. 1C* [19]). This similarity supports the hypothesis that the bound conformation of cyclosporine could preexist in aqueous solution and not be produced by interaction with proteins. Further studies with water soluble cyclosporine derivatives should clarify this point in a near future.

1.2. *Introduction to Cyclosporine Metabolites*

The total synthesis of cyclosporine [20] has been achieved about 10 years ago and since then the synthetic approach has been used many times [21–31] to investigate the relationships between structure and biological activity and to determine the active part of cyclosporine [13][14].

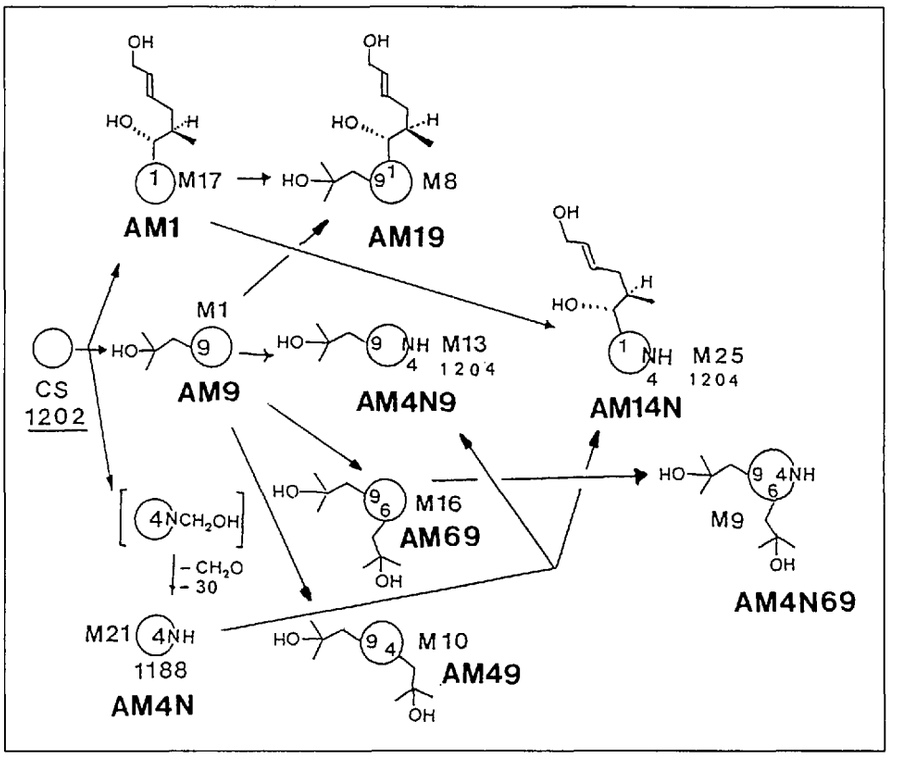
The structure activity relationships of the metabolites were mainly studied with metabolites isolated from urine or bile of patients treated with *Sandimmune* as they were not available from chemical synthesis. They were tested *in vitro* and *in vivo* in animals [32–35].

Metabolites of cyclosporine, the active constituent of *Sandimmune (Sandoz Pharmaceuticals)* are produced by enzyme oxidation (cytochrome P 450-III [36][37] of the cyclosporine molecule at three main points, at residues 1, 4N, and 9. The other metabolites can be interpreted as further oxidation products of these primary metabolites (oxidation at residues 4 and 6, *Fig. 2*). It has been shown [38–42] by incubation of cyclosporine with rat liver microsomes that the first oxidation products are the primary metabolites coded AM1 (M17), AM9 (M1), and AM4N (M21) [43] [44] (*Scheme 1*), which are oxidized in positions 1, 9, and 4N, respectively. Further oxidation of these metabolites could give rise to AM19 (M8, oxidized in positions 1 and 9), AM49 (M10, oxidized in positions 4 and 9), and AM4N9 (M13, oxidized in positions 4N and 9). A similar oxidation pattern is found in human plasma; in human urine, two further man metabolites, both oxidized in position 6, AM69 (M16, oxidized in positions 6 and 9) and AM4N69 (M9, oxidized in positions 4N, 6, and 9) are found which may be considered as further oxidation products of AM9 (M1) and AM4N9 (M13), respectively.

The structure of AM4N9 (M13) [39] has been proposed [38][44], because this structure was the only missing one from all the expected secondary metabolites oxidized in position 9. To confirm the validity of this hypothesis and the structure of this metabolite, a total synthesis of metabolite AM4N9 was undertaken, and a comparison of the synthetic AM4N9 with an authentic sample of M13 isolated from urine [38][39] definitively established its structure as being that of Leu-4-(γ-OH)MeLeu-9-cyclosporine. The description of the synthesis of metabolite AM4N9 is a part of this paper.

From the three primary metabolites AM1, AM9, and AM4N, the synthesis of the first one using cyclosporine as starting

Scheme 1. *Some Metabolites of Known Structures: Possible Ways of Production*



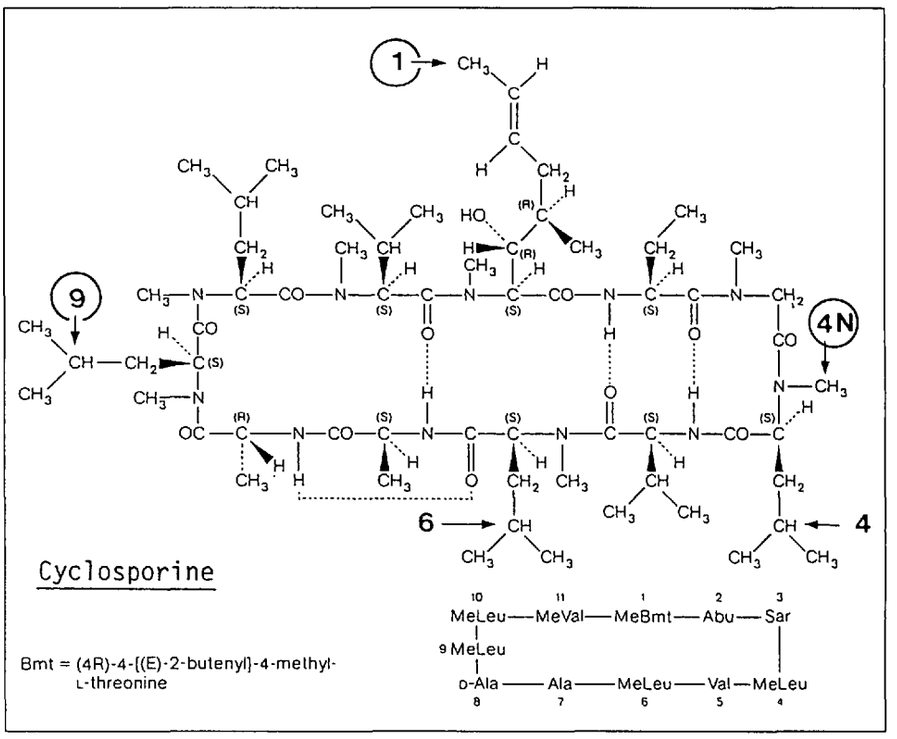
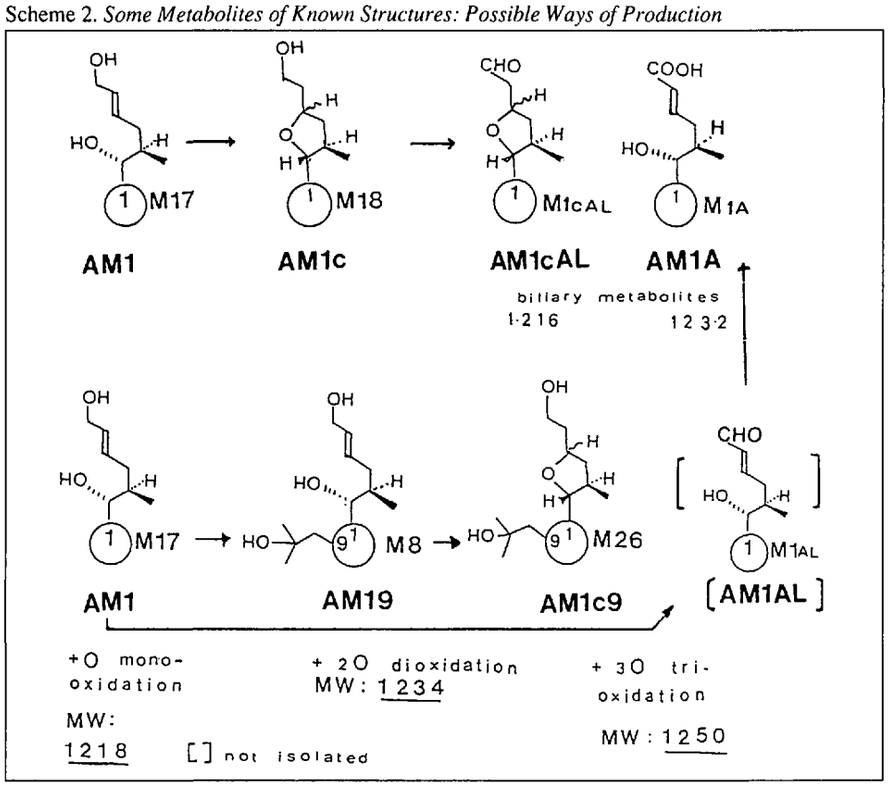
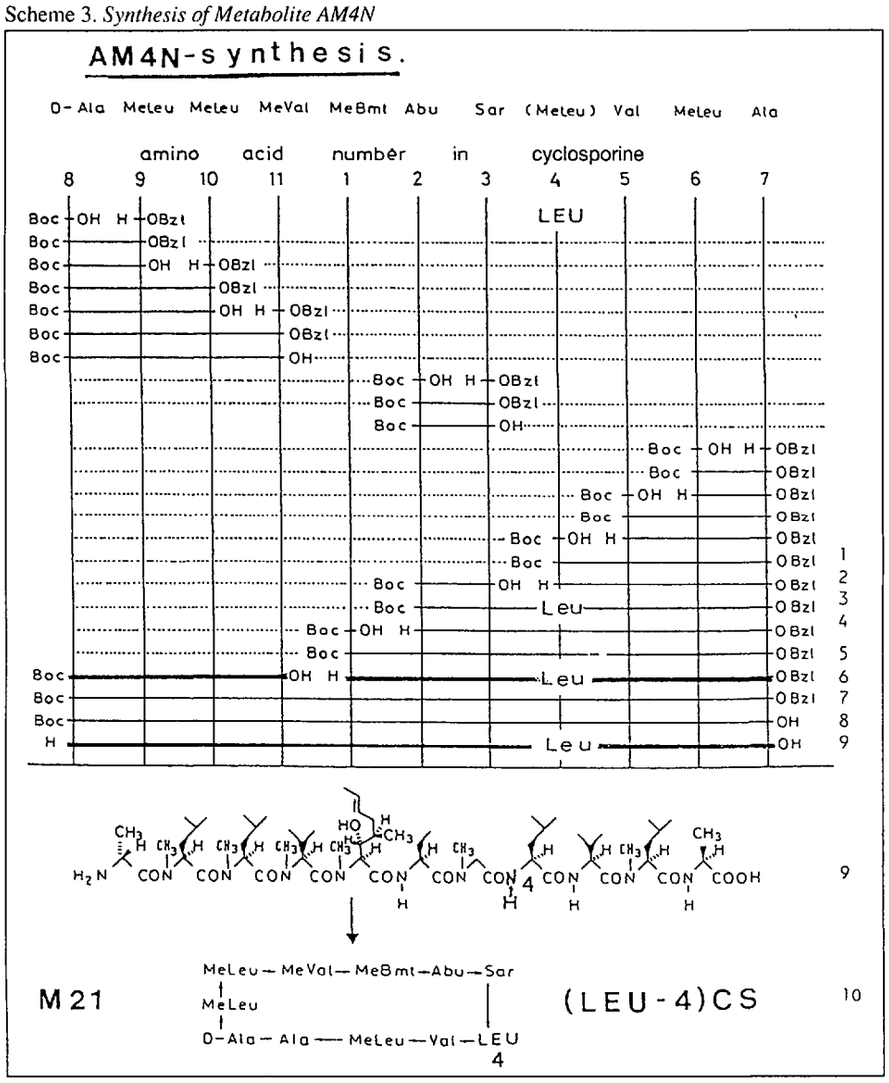


Fig. 2. *1, 4N, 9: Three main points of attack for cytochrome P 450; 1, 4N, 4, 6, 9: five points of attack for cytochrome P 450*

Scheme 2. *Some Metabolites of Known Structures: Possible Ways of Production*



Scheme 3. *Synthesis of Metabolite AM4N*



material has already been mentioned [44], and an alternative synthesis has been recently described [45]. The total synthesis of the two other primary metabolites, (γ-OH)MeLeu-9-cyclosporine (AM9) and Leu-4-cyclosporine (AM4N), will be described prior to the synthesis of the secondary metabolite AM4N9 whose preparative strategy makes use of the two previous ones.

The biliary metabolites AM1AL (7'-CH=O) [46] and AM1A (7'-COOH) [41] are derivatives of AM1 (7'-CH_2OH) obtained by further oxidation [44]. AM1c (3',6'-cyclic ether, 7'-CH_2OH) is a derivative of AM1 obtained by cyclization of the 3'-OH group with the C(6')=C(7') bond of the (8'-OH)MeBmt residue.

The potential primary cyclosporine metabolite AM4 (γ-OH)MeLeu-4-cyclosporine, obtained by enzymatic oxidation of cyclosporine [44][47], has not yet been isolated from the many minor oxidation products found in plasma and urine of patients treated with *Sandimmune*.

The structures of the metabolites which are known today are summarized in the *Schemes 1* and *2*.

2. *Synthesis of Cyclosporine Metabolite AM4N (M21: Leucine-4-cyclosporine* (10) (*Scheme 3*)

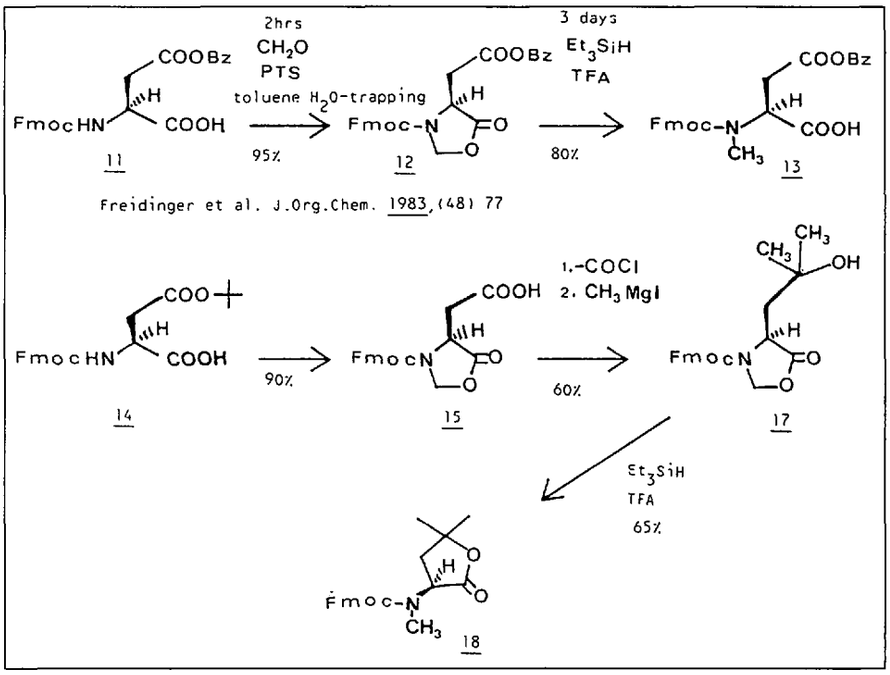
For the preparation of the cyclosporine metabolite Leu-4-cyclosporine (AM4N) (**10**) a L-leucine residue must be introduced in position 4 of cyclosporine instead of *N*-methyl-L-leucine. The synthesis of Leu-4-cyclosporine followed the strategy developed for the synthesis of cyclosporine [20]. Boc-L-leucine was used instead of Boc-MeLeu-OH for the preparation of Boc-Leu-Val-MeLeu-Ala-OBzl (**1**). This tetrapeptide was deprotected with CF_3COOH (TFA) at low temperature (–15°) to produce H-Leu-Val-MeLeu-Ala-OBzl (**2**) which was coupled with Boc-Abu-Sar-OH to give the protected hexapeptide Boc-Abu-Sar-Leu-Val-MeLeu-Ala-OBzl (**3**). The synthesis of the undecapeptide precursor of Leu-4-CS (**10**) followed step by step the procedure used for the preparation of the linear precursor of cyclosporine [20]. After *N*-deprotection of the hexapeptide **3** with TFA to produce **4**, Boc-MeBmt-OH was introduced, the protected heptapeptide **5** isolated and deprotected with TFA to produce H-MeBmt-Abu-Sar-Leu-Val-MeLeu-Ala-OBzl (**6**). This heptapeptide **6** was coupled with the tetrapeptide Boc-D-Ala-MeLeu-MeLeu-MeVal-OH [20] using *Castro* reagent Bt-OP(NMe_2)$_3^+$ PF_6^- in presence of *N*-methylmorpholine to produce the protected undecapeptide Boc-D-Ala-MeLeu-Me-

Leu-MeVal-MeBmt-Abu-Sar-Leu-Val-MeLeu-Ala-OBzl (**7**) which was then deprotected (0.2N NaOH) to **8** and TFA to **9** and cyclized with *Castro* reagent in presence of *N*-methylmorpholine, following the same procedure as described for cyclosporine. The desired (L-leucine-4)cyclosporine (**10**) was isolated and found to be identical in all respects to the metabolite AM4N (M21) isolated from urine [38][39], not only on comparison of all the usual physical and spectroscopic characteristics (IR, NMR, MS, TLC, HPLC) but also on comparison in several biological assays [48].

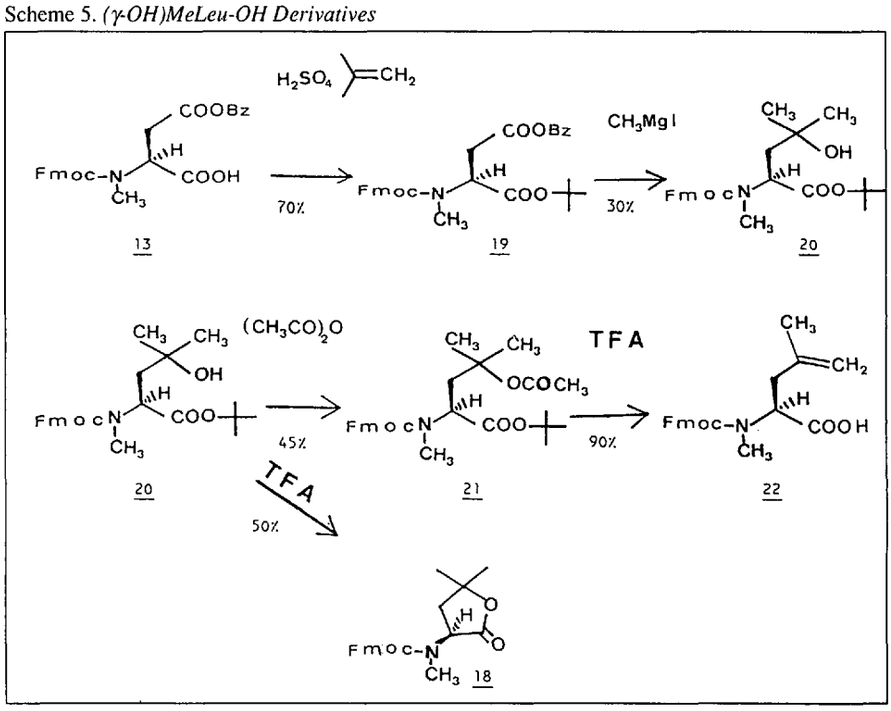
3. *Sythesis of Cyclosporine Metabolite AM9 (M1): (γ-OH)MeLeu-9-cyclosporine* (33)

For the synthesis of (γ-OH)MeLeu-9-cyclosporine a protected *N*-methyl-(4'-OH)-L-leucine is necessary. When Fmoc-(4'-OH)MeLeu-OH was prepared, this derivative cyclized to the corresponding lactone **18** (*Scheme 4*).

Scheme 4. *From Aspartic Acid to (γ-OH)MeLeu-OH Derivatives*



Scheme 5. *(γ-OH)MeLeu-OH Derivatives*



3.1. *Preparation of Fmoc-(4'-OH)MeLeu-lactone* (18)

Fmoc-MeAsp(β-OBzl)-OH (**13**) could be easily produced in two steps according to *Freidinger*'s procedure [49] using Fmoc-Asp(β-OBzl)OH (**11**) as starting material. Reaction of Fmoc-Asp(β-OBzl)OH (**11**) with formaldehyde in acidic toluene produced the *N*-Fmoc-5-oxo-oxazolidine intermediate **12** (95%). Treatment of **12** with Et_3SiH in TFA gave Fmoc-MeAsp(β-OBzl)OH (**13**) (80%). When using Fmoc-MeAsp(δ-OBzl)OH (**14**) as starting material and working as for the preparation of **12**, the *N*-Fmoc-5-oxo-oxazolidine acid **15** was obtained in 90% yield. This acid **15** was then converted with $SOCl_2$ (1.5 equiv.) into the corresponding acid chloride **16** which after evaporation was reacted as crude product with an excess of *Grignard* reagent (10 equiv. CH_3MgI) to produce the *N*-Fmoc-5-oxo-oxazolidine derivative **17** with a yield of 60% over two steps. Reduction of **17** with Et_3SiH in TFA gave after purification Fmoc-(4'-OH)MeLeu-lactone (**18**) in 65% yield.

Efforts to isolate the acid form of this amino acid derivative **18** were not successful.

3.2. *Preparation of Fmoc-(4'-OR)MeLeu-OBu^t (R=H, Ac) and Reactivity of These Amino-Acid Derivatives (Scheme 5)*

Fmoc-MeAsp(β-OBzl)OH (**13**) was esterified with isobutylene in CH_2Cl_2 in the presence of H_2SO_4 (2 d at r.t.) to produce 70% of Fmoc-MeAsp(β-OBzl)OBu^t (**19**). This *N*-Fmoc protected *N*-methylaspartic acid diester **19** was reacted with excess of MeMgI iodide (10 equiv.) at r.t. for 1 h to give 30% of Fmoc-(γ-OH)MeLeu-OBu^t (**20**). Treatment of **20** with TFA at –5° produced the 5-ring lactone **18** which has been previously obtained from **17**. Reaction of the OH group of **20** with Ac_2O in presence of DMAP gave the acetate **21** with a modest yield of 45%. Treatment of Fmoc-(γ-OAc)MeLeu-OBu^t (**21**) with TFA at –5°, following *t*-Bu scissions, led to Fmoc-*N*-methyl-(4-methylidene)norvaline (**22**). Instead of trying harder to replace the acetate group of **21** by an adequate hydroxy protecting group stable under acidic conditions, it was decided, at this stage of the work, to prepare the MeAsp-peptide analogues and to use the peptide amino-acid residues as protecting groups against *Grignard* reaction.

3.3. *Preparation of Fmoc-D-Ala-MeAsp(OBzl)MeLeu-MeVal-OBu^t* (26) *(Scheme 6)*

The dipeptide H-MeLeu-MeVal-OBu^t (**23**) was prepared as described by *Wenger*

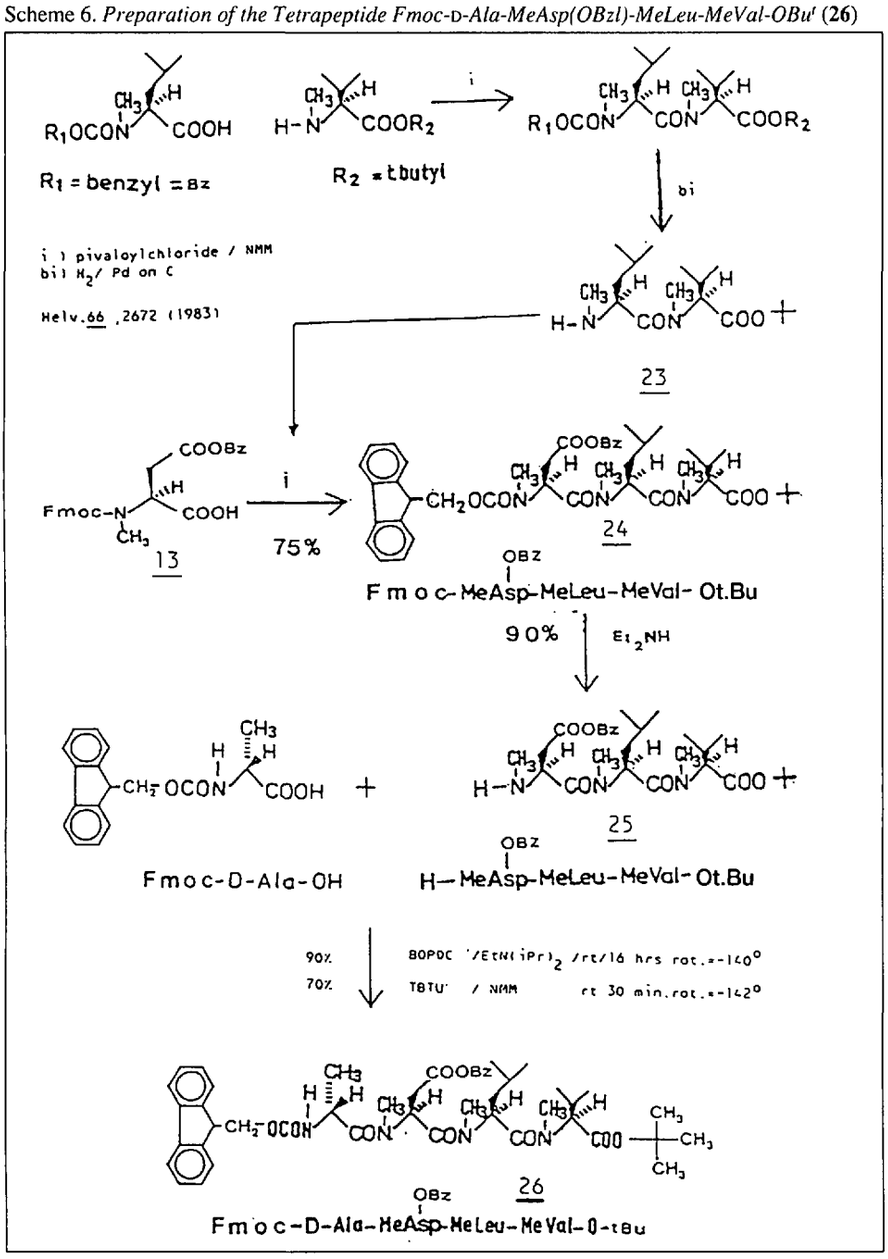
[50] starting from Z-MeLeu-OH and H-MeVal-OBu^t using pivaloyl chloride as a coupling reagent and removing the benzyl-urethane *N*-protecting group with H_2 and Pd on charcoal as a catalyst. This dipeptide **23** was coupled to *N*-Fmoc-*N*-methyl-(*β*-benzyl ester)aspartic acid (**13**) using the same pivaloyl mixed anhydride method to produce Fmoc-MeAsp(OBzl)-MeLeu-MeVal-OBu^t (**24**) in 75% yield. The tripeptide **24** was *N*-deprotected with Et_2NH in MeCN to produce H-MeAsp(OBzl)-MeLeu-MeVal-OBu^t (**25**) (90%). Fmoc-D-Ala-OH was coupled with H-MeAsp(OBzl)-MeLeu-MeVal-OBu^t (**25**) using BOPDC (*N*,*N*-bis-(2-oxo-3-oxazolidinyl)phosphorodiamidic chloride [51][52] as coupling agent (16 h at r.t. in presence of NEt(i-Pr)$_2$) to give Fmoc-D-Ala-MeAsp(OBzl)-MeLeu-MeVal-OBu^t (**26**) with optical rotation $[\alpha]_D^{20} = -140$ (*c* = 1.0 in $CHCl_3$) in 90% yield. A product of similar quality having the same optical rotation ($[\alpha]_D^{20} = -142$, *c* = 1.0 in $CHCl_3$) could be produced in 30 min at r.t. with a not optimized yield of 70% by using TBTU (tetrafluoroborate of (2-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium) [53][54] as a coupling reagent in the presence of *N*-methylmorpholine.

3.4. *Preparation of Fmoc-D-Ala-(γ-OH)MeLeu-MeLeu-MeVal-OH* (28) (*Schemes 7* and *8*)

A solution of Fmoc-D-Ala-MeAsp (OBzl)-MeLeu-MeVal-OBu^t (**26**) in Et_2O was added to 20 equiv. of *Grignard* reagent (MeMgI) in Et_2O and reacted for 1 h at r.t. The reaction mixture was then worked up with 2N H_2SO_4 to produce Fmoc-D-Ala-(γ-OH)MeLeu-MeLeu-MeVal-OBu^t (**27**) (50%). Cleavage of the *tert*-butyl-ester group of **27** succeeded without formation of a 5-ring lactone (similar to **18** [44]) by using TFA at low temperature (first at –15°, then 3 h at –5°). Fmoc-D-Ala-(γ-OH)MeLeu-MeLeu-MeVal-OH (**28**) with $[\alpha]_D^{20} = -130$ (*c* = 1.0 in $CHCl_3$) was obtained in 90% yield after workup at pH 7. This *N*-Fmoc protected tetrapeptide **28** was the desired starting material for coupling with the heptapeptide of the cyclosporine synthesis according to the published strategy [20].

It is of interest to consider that the protection of the acid group of the *N*-protected tetrapeptide Fmoc-D-Ala-MeAsp(OBzl)-MeLeu-MeVal-OH (29), prepared from the *tert*-butylester **26** by TFA treatment at –5°, might not be necessary, since the carboxy group itself of **29** could be used as a protection against *Grignard* reagent. Thus, when a solution of Fmoc-D-Ala-MeAsp(OBzl)-MeLeu-MeVal-OH (**29**) in Et_2O was added to a solution of an excess of MeMgI (20 equiv.) in Et_2O and reacted for 1 h at r.t., then worked up using 2N H_2SO_4 as described for the *tert*-butylester **27**, Fmoc-D-Ala-(γ-OH)MeLeu-MeLeu-MeVal-OH (**28**) was obtained after purification by silica-gel chromatography in 35% in yield.

Scheme 6. *Preparation of the Tetrapeptide Fmoc-D-Ala-MeAsp(OBzl)-MeLeu-MeVal-OBu^t* (**26**)



3.5. *Synthesis of (γ-OH)MeLeu-9-cyclosporine* (33) (AM9) (*Scheme 9*)

Following the procedure developed for the cyclosporine synthesis [20], a 0.05M CH_2Cl_2 solution of Fmoc-D-Ala-(γ-OH)-MeLeu-MeLeu-MeVal-OH (**28**) and of H-MeBmt-Abu-Sar-MeLeu-Val-MeLeu-Ala-OBzl [20] was reacted with 2 equiv. of *Castro* reagent BT-OP(Me$_2$N)$_3^+$ PF$_6^-$ in presence of 2 equiv. of *N*-methylmorpholine for 17 h at r.t. to produce after usual work up and purification Fmoc-D-Ala-(γ-OH)MeLeu-MeLeu-MeVal-MeBmt-Abu-Sar-MeLeu-Val-MeLeu-Ala-OBzl (**31**) in 30% yield. The Fmoc group was cleaved with Et_2NH in MeCN (75%) and the benzyl ester hydrolized with 0.2N NaOH in EtOH at –7° (80%) to produce H-D-Ala-(γ-OH)MeLeu-MeLeu-MeVal-MeBmt-Abu-Sar-MeLeu-Val-MeLeu-Ala-OH (**32**) with an overall yield of 60%. Cyclization of this undecapeptide **32** to (γ-OH)MeLeu-9-cyclosporine (**33**) was achieved in 30% yield by using the condensing agent $(PrPO_2)_n$ in excess and working in low concentration of undecapeptide **32** in CH_2Cl_2 in presence of DMAP as described for the cyclosporine synthesis [20].

The synthetic (γ-OH)MeLeu-9-cyclosporine (**33**) was identical in all re-

spects to the metabolite (M1) isolated from bile and from urine [38][39] not only on comparison of all the usual physical spectroscopic characteristics (IR, NMR, MS, TLC, HPLC) but also on comparison in several biological assays [48].

4. Synthesis of Leu-4-(γ-OH)MeLeu-9-cyclosporine (37, AM4N9) (*Scheme 10*)

For the synthesis of Leu-4-(γ-OH)Me-Leu-9-cyclosporine (**37**) the strategies used for the synthesis of Leu-4-cyclosporine (**10**, AM4N) and (γ-OH)Me-Leu-9-cyclosporine (**33**, AM9) are combined (*Chapt. 2* and *3*).

Fmoc-D-Ala-(γ-OH)MeLeu-MeLeu-MeVal-OH (**28**) was coupled to the heptapeptide H-MeBmt-Abu-Sar-Leu-Val-MeLeu-Ala-OBzl (**6**) containing in this case a leucine residue in position 4. *Castro* reagent was used in presence of DMAP under similar conditions as described for the synthesis of the undecapeptide precursor **32** of the metabolite AM9. The undecapeptide Fmoc-D-Ala-(γ-OH)MeLeu-MeLeu-MeVal-MeBmt-Abu-Sar-MeLeu-Val-MeLeu-Ala-OBzl (**34**) obtained was deprotected and cyclized under the usual conditions described for cyclosporine [20] using $(PrPO_2)_n$ and DMAP to produce Leu-4-(γ-OH)MeLeu-9-cyclosporine (**37**) in an overall yield of 6%. This synthetic Leu-4-(γ-OH)MeLeu-9-cyclosporine (**37**) coded (AM4N9) [43] was identical to metabolite M13 isolated from urine or bile [38][39] on comparison of all the usual physical spectroscopic characteristics (IR, NMR, MS, TLC, HPLC, and $[\alpha]_D$). Thus, the proposed structure for metabolite M13

Scheme 7. *Preparation of the Tetrapeptide Fmoc-D-Ala-(γ-OH)MeLeu-MeLeu-MeVal-OBu^t* (**28**)

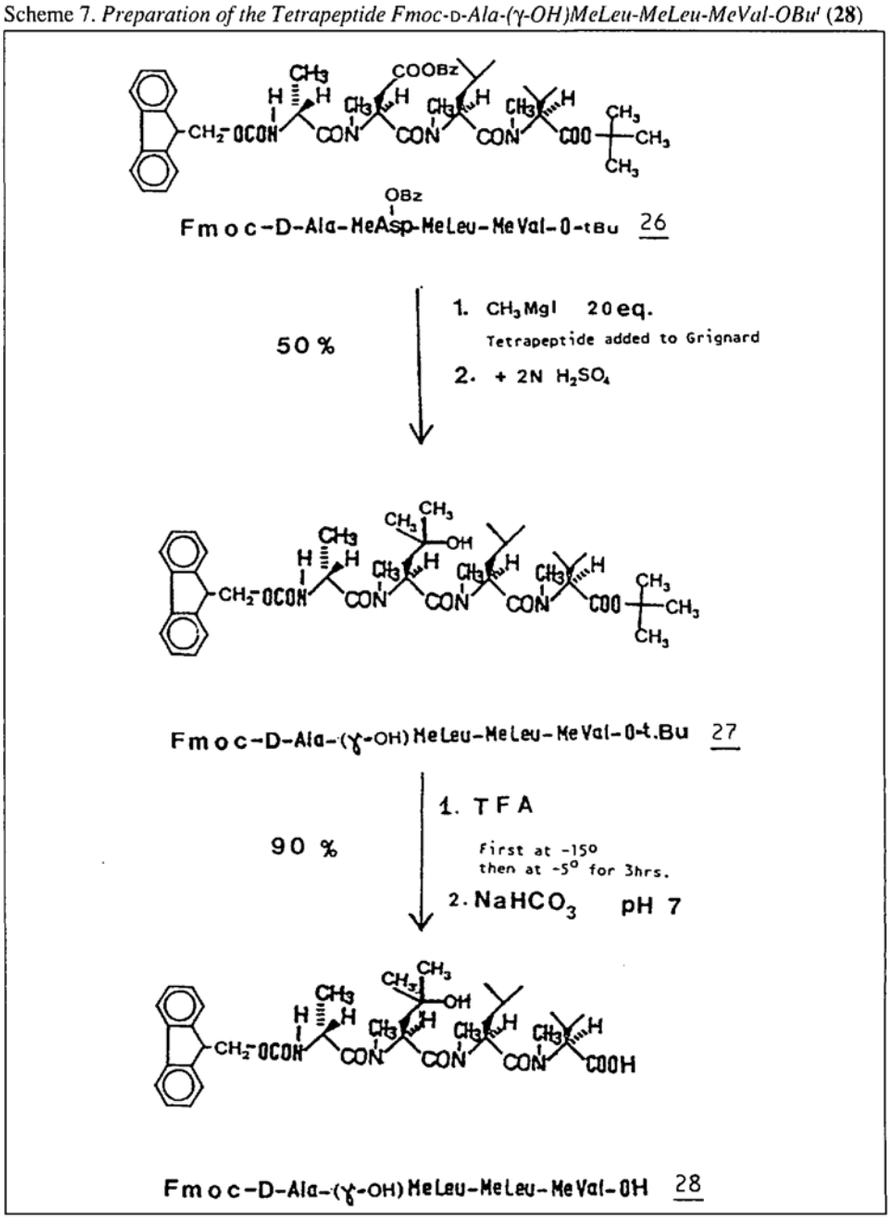
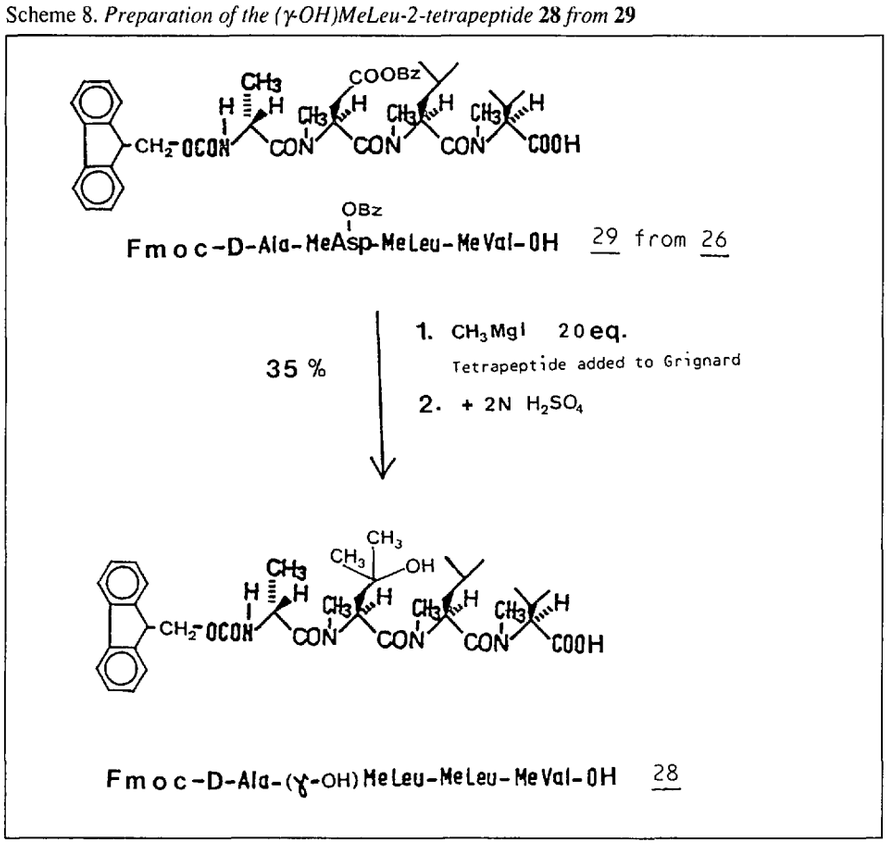


Table 1. *Physical Characteristics of Metabolites AM4N, AM9, and AM4N9 Compared to Cyclosporine and AM1*

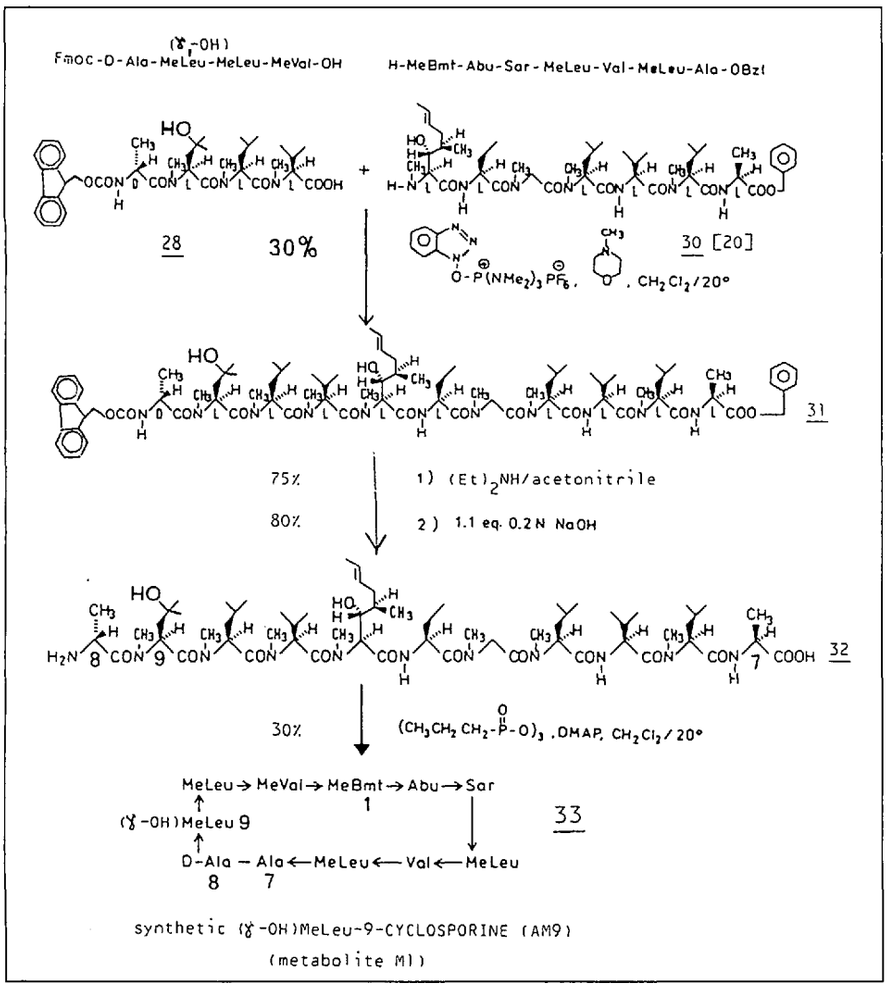
Substance	Conformation in $CDCl_3$	FAB-MS	$[a]_D^{20}$ (c =1.09, in $CHCl_3$)	HPLC/min[a])
Cyclosporine	1 conformation (95% [16])	1202	–244	80
AM1 (8'-OH) MBmt^{-1}	1 conformation (8'-OH bridged with C (4)=O	1218	–269	51
AM4N (**10**)	2 conformations (85 : 15) main conf. = CS	1188	–226	68
AM9 (**33**)	1 conformation similar to CS	1218	–201	53
AM4N9 (**37**)	2 conformations (65 : 35) main conf. = CS	1204	–177	44
	minor conf. = minor conf.of AM4N	Xylene		18

[a]) *SP 8000 BISpectra Physics RP 18* (30 x 4.6 mm) *Brownle* labs.
Column *LC 18* (2 x 150 x 4.6 mm) *Supelco*. Flow: 1.5 ml/min, T = 75°. Eluent: NH_4NH_2COO (0.01M):
MeCN: MeOH 60:30:10 to 42.5:47.5:10 in 40 min, then 15 min constant (t = 55) and to 25:65:10) in 35 min (t = 90 min)
Dr. *G. Maurer*, Preclin Develop., *Sandoz Pharma*, Basel.

Scheme 8. *Preparation of the (γ-OH)MeLeu-2-tetrapeptide* **28** *from* **29**



Scheme 9. *Synthesis of (γ-OH)MeLeu-9-cyclosporine* (**33**)



[44] is now definitively established as being Leu-4-(γ-OH)MeLeu-9-cyclosporine (coded AM4N9).

5. Structure and Conformation of Metabolites AM4N (10), AM9 (33), and AM4N9 (37) in comparison of cyclosporine and AM1

Table 1 shows the influence of the introduction of an OH group into the cyclosporine ring on the polarity of the new derivative as demonstrated by the smaller elution times obtained on HPLC for AM1 and AM9.

The introduction of an OH group did not change the main conformation of cyclosporine, at least not in CDCl3, as only one main conformation was observed by NMR in this solvent for both AM1 and AM9. The change in optical rotation – an increase in negative value for AM1 and a decrease of it in the case of AM9 – might reflect the effects onto the conformation of the cyclosporine ring of the new OH···O=C bridges observed by NMR for these derivatives; (OH–C(4)=O could stabilize the cyclosporine conformation in AM1 ; OH–C(9)=O could modify the cyclosporine conformation in AM9).

Removal of the *N*-Me group in position 4 of cyclosporine seemed to destabilize the cyclosporine main conformation and to favor the formation of a new conformation (15%) in AM4N. AM4N also became more polar. The same effect of the *N*-desmethylation in position 4 was observed in the case of AM4N9 (a *N*-desmethylated AM9 derivative). This effect was even more drastic in case of AM4N9 as demonstrated by the appearance of a more populated new conformation (35%) which was similar to the minor conformation observed in the case of AM4 and by the observation of a smaller elution time on HPLC which reflected a more polar substance.

All these changes in the main conformation of cyclosporine had a negative influence on the immunosuppressive activity of these cyclosporine metabolites which all were much less active than cyclosporine as it will be shown in *Chapt. 6.*

6. Immunosuppressive Activity of Cyclosporine and Some Metabolites (IC_{50} Values)

Table 2 shows the results of the inhibition of cell proliferation in the mixed lymphocyte reaction, on human lymphocytes stimulated with anti-CD3 antibody and in the reporter gene assay [11] [48] by cyclosporine (CSA) and metabolites AM1

[38], AM4N (10), AM9 (**33**), and AM4N9 (**38**) and their cyclophilin (CYP) binding determined in an immunoassay [13].

In the mixed lymphocyte reaction, all metabolites displayed less immunosuppressive activity than cyclosporine. The IC_{50} differed by a factor >10 for AM9, >25 for AM1 and AM4N and >40 for AM4N9.

On human lymphocytes stimulated with anti-CD3 antibody, AM1 displayed the highest inhibitory activity relative to cyclosporine: AM1 differed from cyclosporine by a factor of 10, AM9 by a factor of 30. The other metabolites AM4N and AM4N9 were almost inactive (IC_{50} >1000 nmol/l).

In IL-2 reporter gene assay the most active metabolite AM1 differed from cyclosporine by a factor of 8, AM9 by a factor of 12, and AM4N by a factor of 16. AM4N9 was practically inactive in this assay. In the quantitative immunoassay for CYP [13], it was possible to compare the relative affinity of the metabolites for cyclophilin. The affinity of AM1, AM9, and CSA for cyclophilin binding were not very different and almost identical in the case of AM9. One possibility to explain the discrepancy between CYP binding and decreased immunosuppressive activity could be an impaired uptake of AM1 and AM9 into lymphocytes ([48] and *G. Zenke* unpublished results). The introduction of an OH group in the cyclosporine ring in positions 1 or 9 effected a small conformation change (see *Chapt. 5*) which was not relevant for binding to CYP, but could more consequently influence the uptake of these metabolites by the lymphocytes. The other metabolites AM4N and AM4N9 did not show any relevant binding to CYP.

Scheme 10. *Synthesis of Leu-4-(γ-OH)MeLeu-9-cyclosporine* (**37**)

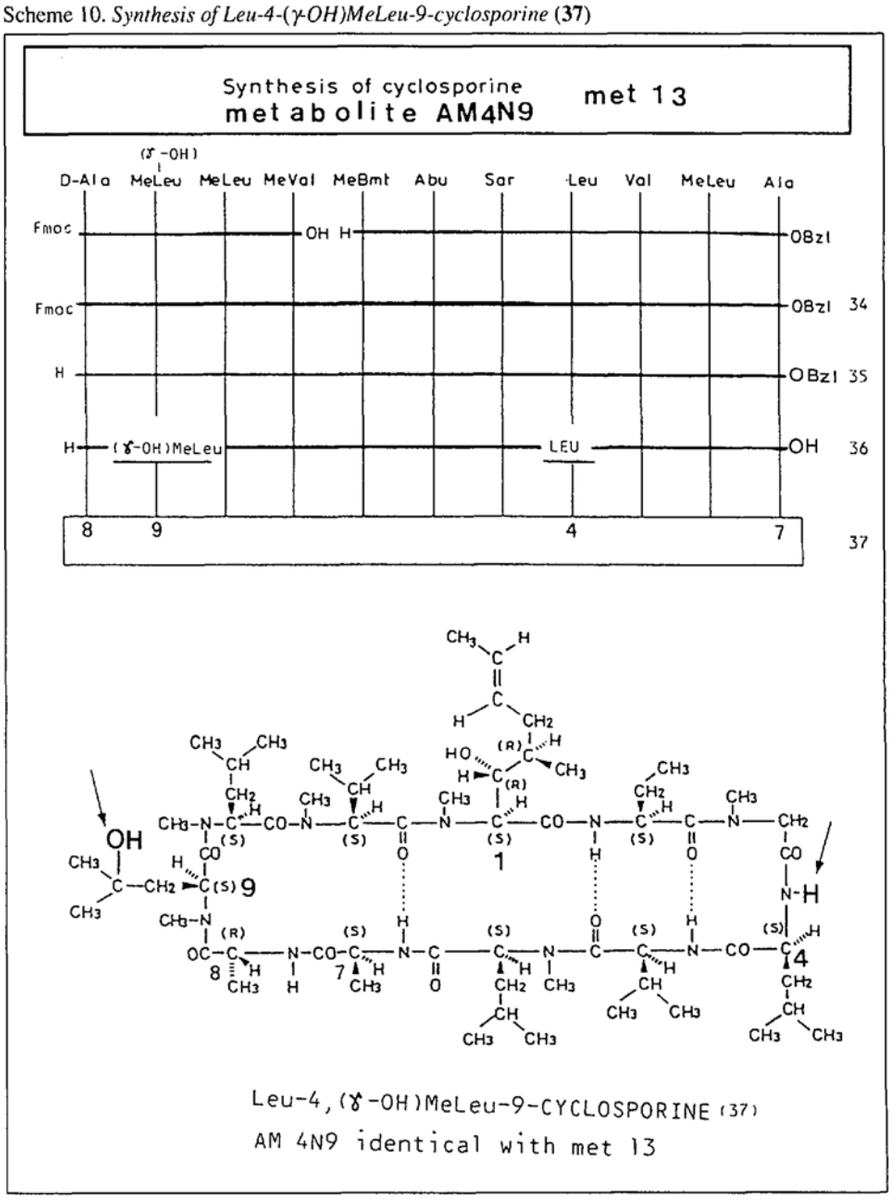


Table 2. IC_{50} *of Cyclosporine and Some Metabolites*

Substance	Murine MLR (ng/ml)	CD3 human PBMC (ng/ml)	IL-2 reporter (ng/ml)	CYP biding ELISA (Km)
CSA	24	10	3	180
AM1[a]) [48]	620	100	24	370
AM4N (**10**)	640	1000	48	> 1000
AM9 (**35**)	280	300	36	250
AM4N9 (**37**)	> 1000	1100	3410	> 1000

[a]) Code means oxidation in position 1 of CS according to [43]. Abbreviations: MLR, mixed lymphocyte reaction; PBMC, peripheral blood mononuclear cells; IL-2, interleukin 2; reporter, reporter gene assay [11]; CYP, cyclophilin; ELISA, enzyme-linked imunosorbent assay, for more details, see [48].

Somewhat puzzling was the finding, that immumosuppressive activity of AM4N was better than it would be expected from its CYP binding value. Probably in the case of AM4N the conformational change observed by NMR (see *Chapt. 5*) had a negative effect for its binding to CYP which could be compensated on the 'effector' domain [11].

7. Conclusion

The present investigations of the metabolites of cyclosporine confirm and extend earlier data from our laboratories.

The total synthesis of metabolites AM4N and AM9 confirms the chemical structures of these metabolites and opens ways to prepare more material for testing. The total synthesis of metabolite AM4N9 definitively establishes the structure of metabolite M13 as being Leu-4-(γ-hydroxy)MeLeu-9-cyclosporine. As a consequence it follows that AM1, AM4N, and AM9 are the main primary metabolites of cyclosporine.

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