REVIEW
OF
LITERATURE
2. REVIEW OF LITERATURE

2.1. Discovery of cyclosporine.

The cyclosporines were originally discovered in 1970 by scientists at Sandoz laboratory in Switzerland while attempting to identify new antifungal agents (Borel, 1982). Crude extracts of two new strains of fungi imperfecti *Cylindrocarpon lucidium* Booth and *Tolypocladium inflatum* Gams demonstrated a narrow spectrum of activity in vitro mainly against clinically irrelevant organisms. However, the antifungal activity was accompanied by a very low degree of toxicity which prompted the investigators to submit the compound for further pharmacological screening including its effect on cell proliferation (Stahlein, 1962), tumor growth and the immune response (Lazary and Stahlein, 1968). In 1972, it was found that the compound was capable of markedly inhibiting hemagglutinin formation against sheep erythrocytes in vivo but appeared to be selective in its immunosuppressive effects. It had no effect on the survival of mice that had been inoculated with L 1210 leukemia cell line. These observations formed the basis for a series of extensive studies of cyclosporine on the immune system.

In 1973, cyclosporine was purified from the fungal extract of *Tolypocladium inflatum* and in 1975 complete structural analysis was
established (Wenger, 1982). In 1980, cyclosporine A (cyc A) was synthesized in the laboratory and its biological activity was similar to that of the cyclosporine A obtained from the fungus (Wenger, 1984).

Early studies of Borel and coworkers (Borel et al., 1976; Borel et al., 1977) clearly established the role of cyc A as a selective immunosuppressor that appeared to act on only a definite subpopulation of immunocompetent cells. Cyc A was found to suppress hemagglutinin formation as well as direct and indirect plaque-forming cell (PFC) response against sheep erythrocytes. Skin allograft survival was prolonged by cyc A and a delayed onset of death was seen in an experimental model of lethal graft versus host disease (GVHD). Cyclosporine reduced the incidence of polyarthritis in rats following administration of Freund's adjuvant and also suppressed disease activity in a rat model of experimental allergic encephalomyelitis (EAE). In contrast to other immunosuppressive agents like azothioprine, it appears to be free of myelotoxicity and did not impair the proliferation of hemopoietic stem cells.

2.2. Chemistry of cyclosporines:

A total of 25 natural cyclosporines have been isolated so far (Traber et al., 1977a,b, 1982). The structures of the congeners have
been determined by spectroscopical evidence, hydrolytic cleavage and identification of the amino acid profile, as well as by chemical correlation reactions, and finally by X-ray analysis. As can be seen from the table 1, exchange of amino acid units occurs in majority of the positions, the exceptions being (so far) positions 3 and 8. Cyclosporines A, B, C, D, and G differ only in the building unit number 2, being L-2-aminobutyric acid in cyc A, L-alanine in B, L-threonine in C, L-valine in D and L-norvaline in cyc G. Another inhomogeneous group is represented by the N-demethylated congeners. N-demethylation may occur at each of the amino acids with the exception of sarcosine. Modification at positions 5 and 7 yield cyc M (L-norvaline instead of L-valine) and cyc V (a second L-α-aminobutyric acid substituting L-alanine). Another striking variation with considerable conformational consequences is encountered in cyc H, which contains N-methyl-D-valine instead of the natural L-epimer.

New analogues of cyclosporines:

The course of cyclosporine biosynthesis by *T. inflatum* is reported to be strongly influenced by an exogenous addition of amino acid precursors (Traber et al., 1989). They have reported that the addition of DL- α-allyl glycine to the medium resulted in its specific incorporation leading to the production of (allyl gly 2)
Table 1: Amino acid composition of Cyclosporines A-Z

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<tr>
<th>Meta-</th>
<th>Amino acid composition</th>
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<td>1  2  3  4  5  6  7  8  9  10 11</td>
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<tr>
<td>Cy A</td>
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<td>MeLeu Nva Ser MeLeu Val MeLeu Ala D-Ala MeLeu MeLeu MeVal Ca&lt;sub&gt;141&lt;/sub&gt;H&lt;sub&gt;121&lt;/sub&gt;N&lt;sub&gt;110&lt;/sub&gt;O&lt;sub&gt;12&lt;/sub&gt;</td>
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Cyclosporine A.

Cyc A is a homodetic cyclic undeca- or decapeptide with a molecular formula of $C_{62}H_{111}N_{11}O_{12}$ and a molecular weight of 1202. It has a rather rigid conformation in the crystalline state as well as in solution (Drevfuss et al., 1976; Petcher et al., 1976). The structure of cyc A is given below.
Cyc A molecule contains eleven amino acid residues. The amino acid residue present in the first position is an unusual amino acid. The unusual amino acid residue in position 1, (4R)-4-(CE)-2-butenyl)-4, N-dimethyl-L-threonine (MeBmt) was first identified in 1980 and stereospecific synthesis of the compound was first demonstrated by (Wenger, 1983). The 10 other amino acids are known aliphatic amino acids. They are L-α-aminobutyric acid in position 2, sarcosine in position 3, N-methyl-L-leucine in positions 4, 6, 9 and 10, L-valine in position 5, L-alanine in position 7, D-alanine in position 8, and N-methyl-L-valine in position 11.

Amino acid residues at positions 1-6 of the backbone adopt an antiparallel beta-pleated sheet conformation which contains three transannular H-bonds and is markedly twisted (Wenger, 1982). The remaining residues 7-11 form an open loop which carries the only D-residue (D-alanine at position 8) and the only cis-amide linkage between two adjacent N-methyl leucine residues (position 9 and 10). The remaining H-bond is of a 3→1 type, which serves to hold the backbone in a folded L shape. Only 4 amide (NH) groups are available for H-bond formation since the remaining seven N-atoms are methylated.

Physiochemical properties of cyclosporine A

Cyc A crystallizes from acetone to give white prismatic needles
which dissolves easily in most organic solvents but poorly in water. Cyc A is a neutral compound as determined by microtitration and electrophoresis experiments. The physiochemical data are listed in table 2 (Ruegger et al., 1976).

Table 2. Physiochemical data of cyc A.

<table>
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<th>Property</th>
<th>Value</th>
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<tr>
<td>Melting point</td>
<td>148-151 °C (cryst. from acetone)</td>
</tr>
<tr>
<td>Optical rotation</td>
<td>[ ] = -244 (in chloroform)</td>
</tr>
<tr>
<td></td>
<td>-189 (in methanol)</td>
</tr>
<tr>
<td>Elemental analysis</td>
<td>C 61.9 H 9.5 N 12.6 O 15.8%</td>
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<tr>
<td>Molar mass (phys.)</td>
<td>1201.842 ± 0.003 (mass spectrum)</td>
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<tr>
<td>Formula</td>
<td>C H N O</td>
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<td></td>
<td>62 111 11 12</td>
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<tr>
<td>Molecular weight</td>
<td>1202.635</td>
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<tr>
<td>Functional groups</td>
<td>1 double bond</td>
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<tr>
<td></td>
<td>1 sec. hydroxy group</td>
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<td></td>
<td>1 amidecarbonyl (C-NMR)</td>
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2.3. Biological spectrum of activity:

The cyclosporines exhibit a rather narrow spectrum of antibiotic activity. Among the yeasts, only a few species are sensitive and no inhibition of bacteria has been observed. Strains of some mucorales, ascomycetes, and fungi imperfecti (e.g. Curvularia lunata, Neurospora crassa, etc.) show differing sensitivity against cyclosporines and inhibition frequently becomes evident as deformation and branching of the growing hyphal tips or simple growth rate reduction in sensitive yeasts (Dreyfuss et al., 1976).

The narrow spectrum of activity of the cyclosporines restricts their use as antifungal antibiotics. However, interestingly, pharmacological investigations have shown that they possess immunosuppressive and antiphlogistic action (Borel et al., 1976) which makes them as potential candidates for transplantation therapy. Also, the very low myelotoxicity associated with these compounds (Britton & Palacios, 1982) makes them the better candidates than the conventionally used corticosteroids and azothioorine.

Structure - Activity Relationships:

Cyc (A to Z) have been tested for antifungal activity as well as in various in vitro and in vivo assays for immunosuppressive activity
such as proliferation of lymphocytes, inhibition of direct plaque forming, cells in mice and rats, hemagglutination test, skin hypersensitivity reaction to oxazolone in mice, etc. (Borel et al., 1976, 1977; Wiesinger and Borel, 1979).

The intact structure of the amino acid 1 (C amino acid) plays an essential role for high immunosuppressive effects. Replacement of this unit by N-methyl-L-leucine (cyc O) or N-methyl-2-amino octanoic acid (cyc 2) as well as the mere absence of the hydroxyl function, like in the desoxy-compounds (cyc F and K), causes a considerable loss of activity. Also, the olefinic double bond in the C9 amino acid seems to play an important role. Dihydro-derivatives are generally less immunosuppressive than their parent compounds. This clearly indicates that the unusual amino acid, MeBmt is intimately involved in the biological activity of the cyclosporine molecule. However, MeBmt alone is not responsible for the immunosuppressive activity since administration of it alone does not produce any immunosuppressive action. In contrast, structural variations in the side chain of the amino acid 2 are tolerated to some extent. Regarding the length and shape of the alkyl residue, cyc A and G with a straight-chained alpha aminobutyric acid or norvaline, respectively, show about equal activity, whereas a shortened or branched alkyl group like in cyc B (alanine) and cyc D (valine) leads to a slight decrease in the activity. Cyc C, which contains a polar threonine in position 2, exhibits a strong immunosuppressive activity.
Modifications are also possible in positions 5 and 7. Replacement of L-valine by the isomeric L-norvaline (cyc M) results in only minor changes in the biological activities as does the variation in cyc V, a homologue of cyc A with a second alpha-aminobutyric acid at position 7 (instead of alanine). On the other hand, N-demethylated congeners in general possess little or no significant immunosuppressive activity. Only marginal activity is encountered with cyc E and W which lack methylation of valine in position 11. In both the cases, an additional hydrogen bond between the amide proton of L-valine and the carbonly group of D-alanine is formed leading to considerable conformational changes in the open loop resulting in a drastic distortion of the ring and complete loss of immunosuppressive activity. All these results indicate that a molecular geometry as in cyc A or a closely congruent backbone conformation is intimately associated in the immunosuppressive activity.

Mode of action of cyclosporine A:

Cyc A is the first of a new generation of immunosuppressive drugs with a specific site of action within the immune system (Borel, 1981). Its action is directed specifically towards the lymphocytes and acts at an early stage of its activation (Borel, 1981). It has a very low degree of myelotoxicity which has made its use in clinical
transplantation attractive. It also suppresses lymphocyte function without damaging the phagocytic activity of the reticuloendothelial system. It does not affect the migratory capacity of the phagocytic cells (McIntosh & Thomson, 1980). Thus, cyc A provides the means for regulating the lymphocyte response without completely suppressing the antibacterial defences of the recipient.

Cyc A affects the function of T lymphocytes and many of its in vivo effects are due to the modification of the T cell function (Dos Reis & Shevach, 1982; Bunjes et al., 1981; Hess et al., 1982 a,b; Orosz et al., 1982). Many studies have demonstrated that cyc A inhibits the proliferative response and the generation of cytotoxic T lymphocytes (CTL) in the mixed lymphocyte response (MLR). Similarly, the lymphoproliferative response to mitogenic stimulation is also inhibited by this unique drug. The inhibitory effect of cyclosporine is not due to lymphotoxicity, since normal responses could be restored immediately after its withdrawal. The inhibition of CTL generation by cyc A appears to occur at two distinct levels:

(1) by preventing production of interleukin-2 (IL-2), a soluble factor which clonally amplifies the activated CTL.

(2) by inhibiting the precursor CTL (pCTL) from acquiring functional responsiveness.

Studies conducted by Larson (1980) in a lectin-dependent system
showed that cyc A inhibited the acquisition of responsiveness to IL-2, leading to postulate that cyc A inhibits pCTL from acquiring receptors to this growth factor. The results of the study conducted by Hess et al. (1982 a,b) using the human MLR system also supported this hypothesis. Their results suggested that at high levels of cyc A (>500 ng/ml), the pCTL did not develop the ability to respond to IL-2, but did acquire responsiveness at lower (<100 ng/ml) doses. The ability of exogenous IL-2 to restore the proliferative response in guinea pig allogenic MLR was also reported by Dos Reis & Shevach (1982), although cytotoxic T cell activity was not assessed. These results suggest that cyc A could block the induction of functional IL-2 responsiveness in some systems, but not in others.

Bunjes et al. (1981) and Wang et al. (1983) demonstrated that addition of cyc A to lymphocytes stimulated with alloantigen in murine MLR, effectively suppressed the production of IL-2. In addition Bunjes et al. (1981) demonstrated that addition of exogenous IL-1 to cyc A treated cultures of Con A-stimulated mouse cells did not result in production of IL-2, indicating that cyc A rendered the T helper cell refractive to the effects of IL-1. These results were confirmed by Hess et al. (1983) who demonstrated that addition of exogenous IL-1 to the primary human MLR could not overcome the immunosuppressive action of cyc A. Similar studies performed by Palacios and Moller (1981) showed that cyc A caused the IL-2 producing T cell to become unresponsive to the monocyte-derived
lymphokine IL-1. This finding has been further characterized at the clonal level by Palacios (1982) and Britton & Palacios (1982), indicating absence or downregulation of the receptor for IL-1-induced by cyc A.

The effect of cyc A on T helper cell function and lymphokine secretion has not been limited to IL-2 production. Recent studies by Thomson et al. (1983b) demonstrated that cyc A inhibited the production of T cell derived lymphokines affecting macrophage function. Addition of cyc A to cultures of spleen cells stimulated with antigen (Con A or phytohemagglutinin A) resulted in the inhibition of the production of macrophage procoagulant activity and a lymphocyte-derived macrophage chemotactic factor. Cyc A did not, however, inhibit the response of the macrophage to these soluble factors. Further studies demonstrated that cyc A was able to inhibit the production of migration inhibitory factor by human lymphocytes stimulated with ConA (Thomson et al., 1983). In addition, a recent report has demonstrated that this novel immunosuppressive agent also inhibits the production of IL-3 (Lafferty et al., 1983). Other studies have demonstrated that cyc A inhibits the production of gamma interferon by lymphocytes that were stimulated by mitogen and/or alloantigen (Abb et al., 1982; Kalman and Klimoel, 1983; Reem et al., 1983). On the other hand, production of alpha or beta interferon by human and mouse lymphocytes or fibroblasts was not affected by this agent (Kalman and Klimoel, 1983).
Elliott et al. (1984) have reported that the effect of cyc A on the interleukin production was mediated by its inhibitory effect on the induction of IL-2 mRNA. These results were confirmed by Granelli-Piperno et al. (1984) who demonstrated that cyc A blocks the induction or production of active lymphokine mRNA in human and murine cell lines. Even though lymphokine production and lymphokine mRNA were inhibited in these studies, total protein synthesis and cell proliferation were not inhibited, suggesting that cyc A can selectively inhibit cellular biochemical functions while not affecting other constitutive processes. Although these results provide information regarding the action of cyc A on lymphokine production, it remains unknown whether the same mechanism also accounts for the ability of cyc A to inhibit T cell activation.

Pharmacokinetics and metabolism.

Absorption.

Cyc A is poorly soluble in aqueous media but highly soluble in less polar solvents (Cavanak and Sucker, 1986). Oral administration of 600 mg of cyc A leads to a maximum serum concentration of 250-1250 ng/ml (mean 538 ng/ml) within 3-4 h after an initial lag time of 0.65 h (Beveridge et al., 1981). The intestinal lymphatic absorption studies by Ueda et al. (1983) in thoracic-duct-cannulated
rats indicate that this mechanism does not play a major role in the absorption of cyc A.

The effect of food intake on the absorption of cyc A administered orally is controversial. While there are reports that the total amount absorbed was significantly reduced by concomitant food intake in patients treated in end stage renal failure, there are reports indicating that the rate and extent of absorption were not affected in normal subjects by concomitant food intake.

Distribution in the blood.

Lemaire & Tillement (1982) and Niederberger et al. (1983) have reported that in the concentration range of 25-500 ng/ml, cyc A is distributed at approximately 20% in the plasma, and 70% in the erythrocytes. At higher concentrations, (more than 500 ng/ml) there is a sharp decrease in the fraction bound to the erythrocytes and a corresponding increase in the plasma level. Also, the distribution of cyc A between plasma and erythrocytes is temperature dependent (Howe & Smith, 1983 and Wenk et al., 1983). When the temperature is decreased from 32°C, it binds more to the erythrocytes. The binding of cyc A to the erythrocytes is rapid and reversible and saturable (Niederberger et al., 1983 and Britton et al., 1984). In the erythrocytes, 80% of cyc A is associated with the cellular contents and it is not bound to carbonic anhydrase and only
moderately bound to hemoglobin (Nussbaumer, 1984). Cyc A also binds to human lymphocytes; however, the binding to leukocytes is less when compared to erythrocyte binding (Ryffel et al., 1982 and LeGrue et al., 1983).

Binding of cyc A to plasma proteins is independent of the concentration but varies with temperature. As the temperature is increased from 4 to 37°C, the binding of cyc A increases from 73% to 97%. Among the different classes of proteins, it is found that 80% of cyc A is associated with lipoproteins and very little binding is observed with albumin. The major lipoproteins involved in the binding are high density lipoprotein (HDL) and low density lipoprotein (LDL) which bind more than 80% of the plasma cyc A. The amount of cyc A bound to chylomicrons and very low density lipoproteins (VLDL) is very low (Mraz et al., 1983).

Tissue distribution.

As cyc A is highly lipophilic, fat contains the highest concentration (Atkinson et al., 1983 and Reid et al., 1983). The tissues which contain high concentrations of cyc A after administration are pancreas, adrenal glands, and to a lesser extent the liver. Very low levels are found in the brain of man (Atkinson et al., 1983) and mice (Boland et al., 1984) indicating that only little cyc A crosses the blood-brain barrier. Cyc A was also
detected in the milk of lactating women on cyc A therapy (Lewis et al., 1983).

Metabolism.

Cyc A is mainly metabolized in the liver (about 99%) and is eliminated through the bile. This is evidenced by (i) the increase in the level of plasma cyc A under simultaneous administration of substances known to inhibit cytochrome P-450 and (ii) decrease in the level of plasma cyc A with the administration of cytochrome P-450 inducers (Sheets & Mason, 1984; Freeman et al., 1984). In human liver microsomal preparations, the degradation of cyc A is NADPH-dependent. The metabolites isolated from the urine (Maurer et al., 1983) indicate that the reactions involved in the biodegradation of cyc A are limited to N-demethylation, hydroxylation and cyclization. The resulting products are sufficiently water soluble for them to be preferentially excreted along with the urine or bile. The transformation sites are limited to four amino acids, namely amino acid 1, 4, 6 and 9. However the cyclic oligopeptide structure is not altered in any of the metabolites. Hydroxylation occurs mainly at -position of the amino acid 1 and the -position of the N-methyl leucines at positions 4, 6 and 9. N-demethylation occurs only on the N-methyl leucine in position 4. N-methyl leucines 6 and 9 are only hydroxylated while amino acid 1 and the N-methyl leucine 4 undergo two biotransformation reactions viz. hydroxylation and
intramolecular ether formation or hydroxylation and N-demethylation. In all these cases the cyclic structure of the oligopeptide remains unaltered.

The degradation products of cyc A have only very low immunosuppressive activity and hence may not contribute to the overall immunosuppressive activity of cyc A. They are also less toxic compared to the parent compound and they possess no nephrotoxicity which is the major side effect associated with the parent compound. Cunningham et al. (1983 & 1984) have shown that the administration of Aroclor 1254 which increases the metabolism of cyc A, resulted in reduced nephrotoxicity. Similarly, administration of ketoconazole which increases when the metabolism of cyc A, resulted in decreased levels of plasma creatinine (Ferguson et al., 1982).

Elimination.

It was found that following intra-venous administration of cyc A, the blood concentration elimination occurred tri-exponentially with half-lives of 0.1, 1.1 and 16 h (Follath et al., 1983). The clearance from whole blood was found to be 0.37 l/h/kg (Follath et al., 1983) while in children it was found to be 40% higher than in adults (Burckart et al., 1985). This explains the need for the higher dose of cyc A required for children than the adults (Burckart et al., 1985 and Klare et al., 1984). The main route of elimination
of cyc A and its metabolites is through biliary excretion and urinary elimination is not of much significance (Wood et al., 1983). It has been reported that the total clearance of cyc and its metabolites was reduced in children with liver failure (Burckart et al., 1984) and in bone marrow transplant recipients with reduced liver function (Yee et al., 1984).

Antiparasitic effect of cyclosporine A.

Cyc A has attracted the attention of experimental parasitologists due to its usefulness as a probe for dissecting the role of T-cells in immunological responses. In 1981, it was found that it possessed antiparasitic effects against murine schistosomiasis (Bueding et al., 1981) and malaria (Thommen-Scott, 1981). More recently, the spectrum of antiparasitic activities of cyclosporine has been found to include toxoplasmosis (Mack and Mc Leod, 1984) and filariasis (Bout et al., 1984a). Trypanosomiasis (Mc Cabe et al., 1985) and giardiasis (Belosevic et al., 1986) however appear to be unaffected. Bueding et al. (1981) have suggested that the specific inhibition of haemoglobinase activity of female worms following administration of cyc A to the host, could account for the antischistosomal effect. However, the drug did not inhibit the haemoglobinase activities of schistosomes in vitro and a direct toxic effect of cyclosporine against schistosomula has not been demonstrated in vitro even in the presence of serum from infected mice. More recently, Bout et al.
(1984b) and Nilsson et al. (1985) have confirmed the protective effect of cyc A against _Schistosoma mansoni_ and corroborated the observation that the drug is most effective against immature stages of the worms during the early stages of infection. In mice treated with cyc A (30mg/kg/d) from day 1 (infection on day 0) to day 3, only 10-30% of schistosomula were recovered by pulmonary perfusion on day 6 compared with controls. Animals in which cyc A caused complete elimination of the parasites from the primary infection were resistant to infection at 45 days. Cyc A treatment (50 mg/kg given subcutaneously for 5 days) in mice simultaneously with infection (_Schistosoma mansoni_) completely eliminated the parasite within 49 days. As the interval between the infection and the treatment was increased, the chemotherapeutic effect was also reduced. Administration of cyc A up to 15 days before infection was found to be highly prophylactic (Thomson et al., 1986) as has earlier been reported by Bout et al. (1986). The few worms that survived were stunted and sexually immature at 45 days post infection. Both the number of eggs and the granulomatous responses were reduced by cyc A. Probably, the drug may have a direct effect on parasite reproduction (Thomson et al., 1986).

Munro and McLaren (1990) have reported that the schistosomicidal effect of cyc A is due to a metabolite of the drug though the major metabolites are known to possess limited immunosuppressive activity (Chabannes et al., 1987; Rosano et al., 1987; Schlitt et al., 1987).
Further evidence for the partition between immunosuppressive and schistosomicidal effects of cyc A is described by Chappel et al. (1987) who described the prophylactic and immediate schistosomicidal effects of cyc. The schistosomicidal effect of cyc A remains longer than its elimination, which suggests that a lipophilic metabolite of it may be incorporated into the schistosome and adversely and permanently affect the function of a critical metabolic pathway or enzyme system of the parasite (Munro and Mc Laren, 1990).

Cell mediated immune responses are considered to be important for the development of immunity to malaria, especially the asexual blood stages (WHO, 1985). It has been suggested by Quinn et al. (1986) that there might be a higher incidence of malaria in patients suffering from acquired immunodeficiency syndrome (AIDS) in endemic areas of Africa.

In a study conducted by Somasundaram et al. (1989) in mice, it has been reported that cyc A administration at 3 & 6 mg/kg/d to mice infected with $1.5 \times 10^8$ Plasmodium berghei-parasitized red blood cells (PRBC's), reduced the degree of infection. Complete elimination of the parasites from the peripheral blood was achieved by day 6 after infection while the control animals developed high parasitaemia and were dead by day 12 after infection. No recrudescence of the infection was noticed in the treated animals even after the withdrawal of cyc A.
Depression of humoral and cell mediated immunity or both exacerbate(s) parasitic infections such as amoebiasis (Armon, 1978), toxoplasmosis and malaria (Loke, 1982). However, in the study conducted by Somasundaram et al. (1989) and Nickell et al. (1982) malaria infected mice treated with cyc A recovered from the infection. As cyc A is known to be a cytotoxic drug it may be concluded that the drug acts directly on the parasite and kills it.

Toxicity of Cyclosporine.

Unlike other immunosuppressive drugs like azothioorine and other corticosteroids, cyc A has a very low myelotoxicity, but it has other definite and reversible organ toxicities (Britton & Ronald Palacios, 1982). The oral LD50 dose for mice is 2.3 g/kg, which is well over 50 times the therapeutic dose required for effective immunosuppression in this species. While lethal doses caused severe hepatotoxic and nephrotoxic lesions with necrosis as discerned by necroscopy, sublethal doses caused only very minor histological changes (Borel, 1981).

In man, reversible nephrotoxicity (Hows et al., 1981) and hepatotoxicity (Klintmalm et al., 1981) has been reported. Nephro-toxicity mainly involves proximal tubular lesions and is associated with increased levels of serum creatinine and blood urea nitrogen (BUN). This is an obvious drawback in kidney
transplantation but these changes are reversible and hence it is possible to control them by adjusting the dosage of cyc A (Hows et al., 1981). Hepatotoxicity is less frequent (Klintman, 1981) and is evident from changes in various liver enzymes like serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT) and alkaline phosphatase and also by changes in the level of serum bilirubin. But cyc A in combination with prednisolone has been used successfully as an immunosuppressant in the transplantation of liver (Starzl et al., 1981b).

Besides these effects, hirsutism (especially on the face), gingival hyperplasia and tremor have been reported, notably at high serum concentrations (Laupacis, 1983).

2.4. Production of cyclosporine A.

Microorganisms which produce cyclosporines.

The fungal genus, Tolypocladium, first described by Gams (1971), belongs to the class Fungi-imperfecti occurring in soil or litter habitats. Of the nine species accepted in Tolypocladium, four are pathogens of terrestrial invertebrates, and one species is often isolated from myxomycete sporangia. Relatively little is known about
the biology of individual *Tolypocladium* species. The species are characterized by white, hyaline or bright coloured, relatively slow-growing cottony colonies (Bisset, 1983; Samson and Soares, 1984). *Tolypocladium* is often characterized as a Beauveria-like genus based on the similarities in conidiophore cells and conidia. Von Arx (1986) has suggested that *Tolypocladium* should be reclassified as *Beauveria*, although this is currently a point of contention (Samson et al., 1988).

Chun and Agathos (1989) have reported the production of pink pigment along with cyc A by *T. inflatum*. Yellow pigment was reported by Weiser and Matha (1988). Aarnio and Agathos (1990) have reported the isolation of colony types of *T. inflatum*: morphologically normal white, red, and orange colonies and morphologically diverse tiny brown colonies. Each colony type was tested for its pigment and cyclosporine production in shake flask cultures. They have reported that the normal white *T. inflatum* and the brown variant developed into yellow broths in liquid cultures, whereas orange and red colonies generated dark brown and black broths, respectively. The brown variant grew extremely intensely compared to the original strain, but had low volumetric and specific production of cyc A. Conversely, the biomass concentration of the orange and red variants were low. The volumetric production of cyc A of the orange variant was equivalent to the original strain. However, the red variant reached almost 50% higher cyc A titre. For the orange and the red variants, the
specific production of cyc A was approximately two and three times that of the original white colony respectively. The final pH of the brown variant was markedly lower than the original strain. The pH of the broths of the red and orange strains were the highest observed. In general, they concluded that higher the final pH, the higher the specific cyc A levels obtained. The differences in growth and in final pH indicate that there may be different primary metabolic pathways operating among the variants.

In addition to cyc A, the fungus T. inflatum produces a plethora of minor metabolites of the same structural type as shown in Table 1.

The production of cyc A by Beauveria nivea has been reported by Margaritis and Chahal (1989). They have reported that in a fructose based medium (3%), a maximum yield of 170mg/l was obtained in 8 days of fermentation.

Nakajama et al. (1989) have reported the production of cyclosporine by many species of the fungi belonging to the genus Neocosmospora: _N. vasinfecta_ var. _africans_ IFO 7590, _N. vasinfecta_ var. _vasinfecta_ IFO 8963, IFO 8964, IFO 8965, IFO 8966, IFO 30062, IFO 31377 and _N. boninenses_ NHL 2919. They have reported 0.78 mg of cyc A per litre of the culture filtrate of IFO 31377 and 19 mg of cyc A per litre of the medium (from the mycelial mats) from NHL 2919. It was also reported that IFO 8966 was a unique strain in that it
produced mainly cyc C at a concentration of 1.7 mg/l of the culture and they have suggested that it may be due to the qualitative and quantitative difference in the mycelial amino acid pool. *N. vasinfecta* NHL 2298 was the only strain in which N-acetyl C 9 amino acid was secreted in the culture filtrate.

*T. inflatum* grows well on yeast-malt agar at a temperature of about 24°C. For submerged cultivation a medium containing glucose and casein hydrolysate and peptone supplemented with mineral salts is being used. After 10-12 days of fermentation, the biomass is harvested by centrifugation of the broth (Dreyfuss et al., 1976) and the product isolated and purified from the broth. A general outline of the various steps employed in the isolation and purification of cyc A and C is given in fig.1.
Fig. 1.

Isolation and purification scheme for the production of cyc A_2.

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culture broth

extraction with organic solvents

centrifugation

removal of lipids

crude extract

repetitious chromatography
(silica gel, sephadex LH-20, alumina)

crystalline cyclosporine A  
crystalline cyclosporine C
Effect of precursors on the synthesis of cyclosporines.

Kobel and Traber (1982) have reported that feeding of specific precursor amino acids to the cultures of \textit{T. inflatum} shifted the rate of synthesis of different cyclosporines. It was shown that addition of DL-amino butyric acid at 8 g/l resulted in the exclusive production of cyc A and addition of L-norvaline (8g/l) resulted in the accumulation of cyc G (91% of the total cyclosporines). Addition of L-threonine (8g/l) resulted in an overall increase of the total cyc level (by 5 fold) with a specific yield of 59% cyc A and 41% cyc C. Addition of L-valine (8g/l) was also shown to increase the yield of total cyclosporine (by 5.7 fold) with a specific yield of 43% cyc A, 20% cyc C and 37% cyc D.

An amount of 8g/l of the precursor amino acids were found to be optimum for the above described directed biosynthesis of the cyclosporines. These results provide further evidence for the low specificity in the non-ribosomal biosynthesis of peptides in fungi as in the case of ergot alkaloids (Kobel and Sanglier, 1978; Beacco et al., 1978).

A wide range of carbon sources were studied by Agathos et al. (1986) for the enhanced production of cyclosporine. They have reported that 3% (w/v) sorbose yielded the highest productivity of cyc A (105.5 mg/l) after 10 days of fermentation at 27°C with
shaking. Sequential addition of maltose was reported to enhance the total production of cyc A.

Agathos et al. (1987) have reported the influence of different carbon sources and aeration on the yield of cyclosporine using the strain Tolypocladium inflatum. They have reported that sorbose, glucose, maltose, fructose and cellobiose favoured good cyc A production. Under shake flask condition a medium containing 5% glucose or maltose yielded 50 mg/l of cyc A on day 10 without any lag period between growth and production phases. However, under aerated and stirred conditions, the same medium yielded 4.5 times increased production of cyc A. Also, they have reported that addition of maltose as a sequential carbon source increased the production irrespective of the initial carbon source.

2.5. Biosynthesis of cyclosporine.

Cyc A represents a cyclic, partially N-methylated undecapeptide containing the unusual amino acids, L-2-amino butyric acid, D-alanine, and the hitherto unknown (2S, 3R, 4R, 6E)-3-hydroxy-4-methyl-2-methylamino-6-octoic acid (C9-amino acid). These facts, together with the broad pattern of congeners, indicate a non-ribosomal pathway involving a multifunctional enzyme as established for other small peptides of microbial origin, such as gramicidin S (Kleinkauf and
Koischwitz, 1978) and enniatin (Zocher et al., 1982).

In an independent investigation on the biosynthesis of cyc A under different fermentation condition, Zocher et al. (1984) found that short term feeding of the cultures of \textit{T. inflatum} with \textit{14}$^C$-labelled amino acids leads to a selective incorporation of L-leucine, L-valine, glycine and D & L-alanine into cyc A (and C). Experiments with L-(\textit{C-methyl}) methionine demonstrated that all N-methyl groups originate from methionine.

Zocher and Billich (1987) have reported an enzyme fraction from the mycelium of the fungus \textit{Tolypocladium inflatum} with a molecular weight of 700,000 which is capable of synthesizing different cyclosporines \textit{in vitro} in the presence of the constituent amino acids, ATP, and S-Adenosyl methionine (the methyl donor). This shows that the \textit{in vivo} synthesis of cyclosporine is non-ribosomal and involves only enzyme systems.