8 Immunomodulators

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CONTENTS

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I. Introduction

Immunomodulators are actually natural products of the immune system (Pirofski and Casadevall 2006). The immune system of healthy organism produces a diverse range of metabolites to keep the body in homeostasis condition. An immunomodulator may be defined as a substance, of biological or synthetic origin, which can stimulate, suppress or modulate any of the components of the immune system including both innate and adaptive arms of the immune response (Agawal and Singh 1999). In clinical practice, immunomodulators are classified into three main categories:

1. **Immunosuppressants** are agents that somehow inhibit the immune system. They can be used for the control of pathological immune response after organ transplantation and for the treatment of autoimmune diseases, hypersensitivity immune reactions as well as immune pathology associated with infections.
2. **Immunostimulators** are agents that stimulate the immune system by inducing the activation or increasing activity of any of its components. They enhance the body's resistance against allergy, infection, cancer and autoimmunity.
3. **Immunoadjuvants** are agents used to enhance the vaccine efficacy. This can be also considered as a specific immune stimulator effect.

The industrial importance of immunomodulators is based on their large market value. The market size of immunomodulators was evaluated at US $43 billion in 2006, and is expected to grow at a compound annual growth rate (CAGR) of 13% to reach US $80 billion by 2011 (Research and Market 2007). Although some potential immunomodulating substances can be chemically synthesized and have been successfully tested for modulation of the immune system [e.g. synthetic muramyl dipeptide (MDP) analogues], research activities

in this field have been focused on immunomodulatory active compounds from natural resources. Among them, fungal immunomodulators represent the most interesting group of metabolites. This review outlines the current state of knowledge on fungal immunomodulators used already for different medical applications and discusses the future potential of new compounds of fungal origin.

II. Immunosuppressants

The importance of immunosuppressants arose in the mid of the twentieth century in the context of new developments in organ transplantation. The principal goal of immunosuppressant application in organ transplantation is to minimize the risk of allo-graft rejection or graft-dysfunction by achieving adequate immunosuppression, yet also to ensure that the level of immunosuppression does not contribute to long term morbidity (Patel and Kobashigawa 2008). The first organ transplantation was performed in 1933 when a kidney was transplanted from a cadaver. Total lymphoid irradiation was used for the immune suppression but the tissue was rejected and the patient eventually died. This was followed by the use of corticosteroids as immunosuppressive agents, but unfortunately, these steroids as such did also not give the positive results expected. In the early 1960s, cytotoxic agents such as modified corticosteroids were introduced to suppress the immune system after organ transplantation (Khan 2008). The first clinically used fungal immunosuppressive agent was introduced into the market in the mid of 1980s when cyclosporine became available for clinical applications after getting its approval from the United States Food and Drug Administration (US FDA). This was one of the most important milestones in the history of organ transplantation. Beside their important roles in organ transplantation, immunosuppressive agents are used for other applications, for example, in the prevention of the newborn Rh hemolytic disease (Contreas and DeSilva 1994) or for the treatment of some autoimmune diseases. The chemical structures of the three main fungal immunosuppressive agents (cyclosporine, mycophenolic acid, mizoribine), which all are used in organ transplantation and for other medical applications, are shown in Fig. 8.1.

A. Cyclosporins

Cyclosporins (Cys) are a family of neutral, high lipophilic, cyclic undecapeptides containing some unusual amino acids and having a remarkable spectrum of biological activities. The first member of this class of compounds was named cyclosporine A. To date, more than 30 members of this family of compounds have been isolated from natural resources and were classified as cyclosporins A to Z (CyA–Z; Traber et al. 1982, 1987). CyA was originally described as an antifungal peptide with a narrow spectrum of efficacy. However, the interest in this compound only increased significantly after the demonstration of its specific immunosuppressive activity. In 1983, Sandoz first introduced a cyclosporine-A-based drug, Sandimmun, into the market. The modified form of this drug with increased bioavailability, Neoral, became available on the market in 1994 in form of soft gelatins and oral applicable solutions. Since then, Sandimmun and Neoral have been Novartis' leading pharmaceutical products and these drugs generated a revenue of US $1.216 billion in 1997 (Svarstad et al. 2000). Nowadays, based on market research data, only five members of the cyclosporin family, namely CyA (CAS 59865-13-3), CyB (CAS 63775-95-1), CyC (CAS 59787-61-0), CyD (CAS 63775-96-2) and CyH (CAS 83602-39-5) are commercially available for pharmaceutical applications as immunosuppressive agents.

According to their immunosuppressive mode of action, cyclosporins belong to the specific calcineurin inhibitors. Immunosuppressant activity is mediated through blocking the activation and proliferation of $CD4^+$- and $CD8^+$-T lymphocytes by inhibiting IL-2 production (Siekierka et al. 1989; Shibasaki et al. 2002). Under normal conditions, the binding of major histo-compatibility peptides to the T-cell receptors results in the formation of an activated form of calcium/calmodulin-dependent serine/threonine phosphatase calcineurin. This leads to dephosphorylation and nuclear translocation of the nuclear factor of activated T-cells (NF-AT), Subsequently, NF-AT binds genes encoding pro-inflamatory cytokine IL-2, resulting in an up-regulated gene transcription (Schreiber and Crabtree 1992; Butch 2008). CyA freely crosses lymphocyte membranes and forms complexes with the specific cytoplasmatic binding protein immunophilin cyclophilin A. The CyA-cyclophilin A complex inhibits calcineurin

2-Mycophenolic acid (MPA)

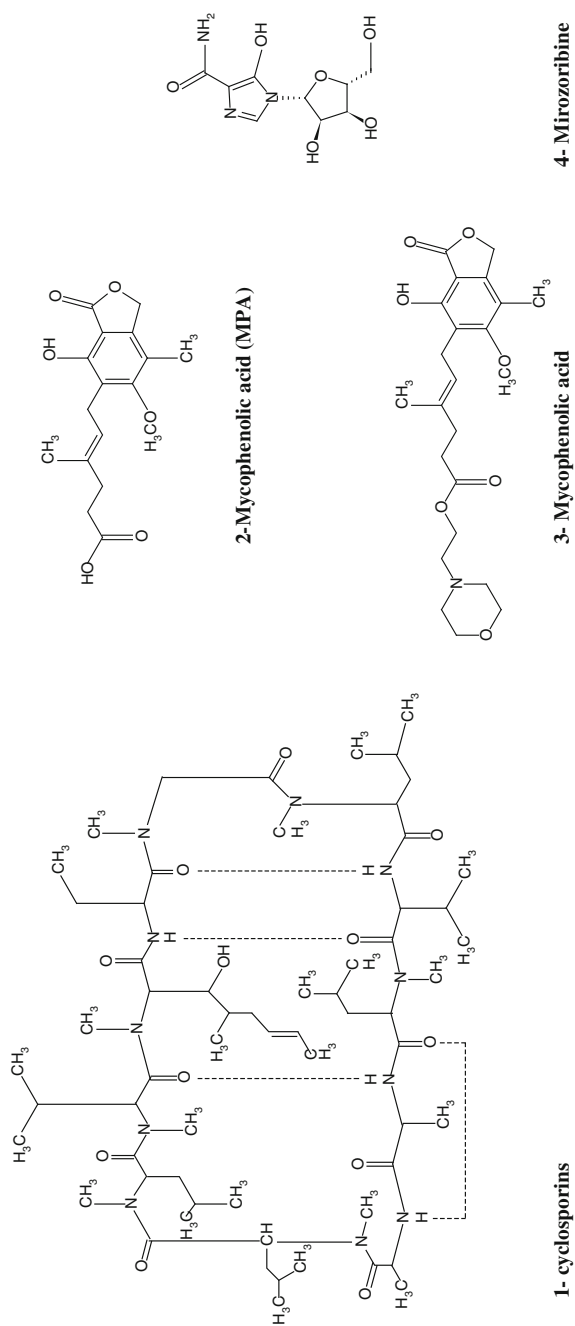**3- Mycophenolic acid**

4- Mirozoribine

1- cyclosporins

Fig. 8.1. Chemical structure of the main clinically important fungal immunosuppressives compounds

activity and the nuclear translocation of NF-AT. This leads to the down-regulation of the pro-inflammatory molecules gene transcription and subsequently halts the production of IL-2 and TNF-α (Jorgensen et al. 2003).

Currently, CyA is approved and used worldwide as an immunosuppressive drug to prolong organ and patient survival after kidney, liver, heart and bone-marrow transplants. CyA is available on the market under the trade name Sadimmune for both oral and intravenous applications. A nano-sized pre-concentrate formulation of CyA (CyA-MEPC or Neoral) exhibiting a better absorption characteristic is used orally in form of solutions or soft-gelatin capsules (Vonderscher and Meinzer 1994; Uchida et al. 2004). Besides the products of the market leader Novartis, several generic formulations are nowadays available and are often referred to as modified CyAs (Alloway 1999).

1. Chemistry

CyA, the most important member of the cyclosporin family, is a cyclic undecapeptide with a 33-membered ring composed of 11 lipophilic aliphatic amino acids, of which four are leucine and three are the non-proteogenic amino acids D-alanine, (4R)-4-[(E)-2-butenul]-4-methyl-L-threonine (Bmt) and L-α-aminobutyric acid. The full chemical name of CyA is 32-ethyl-2-[(E,1R,2R)-1-hydroxy-2-methylhex-4-enyl]-3,6,9,12,14,17,21,27,30-nonamethyl-8,11,20,26-tetrakis-(2-methylpropyl)-5,23-di-(propan-2-yl)-3,6,9,12,15,18,21,24,27,30,33-undecaza-cyclotritriacontane-1,4,7,10,13,16,19,22,25,28,31-undecone. The molecular formula and molecular weight of CyA are $C_{62}H_{111}N_{11}O_{12}$ and 1202.61 g mol^{-1}, respectively. The non-proteogenic amino acids are found at the positions 1 (Bmt), 2 (L-α-amino butyric acid) and 8 (D-alanine). Remarkably, seven of the 11 peptide bonds are N-methylated, which has several important implications. First, the N-methylated peptide bonds and the cyclic structure of the molecule renders cyclosprins stable toward mammalian digestive and systemic proteases. Cyclosporin metabolism in animals and humans is exclusively carried out by cytochrome P450 enzymes catalyzing its oxidative transformation. Therefore, cyclosporins are not only well absorbed when given orally but also characterized by high and long-lasting

plasma levels. A second consequence of the N-methylation pattern is rigid conformation in the non-polar environment characterized by intramolecular hydrogen bonds being oriented towards the hydrophobic environment (Kallen et al. 1997).

Plain cyclosporin is difficult to crystallize on its own and therefore, was initially analyzed as crystalline iodo-cyclosporin. The structural analysis of such CyA crystals by X-ray diffraction revealed a rigid conformation (Loosli et al. 1985). The rigidity can be attributed to a number of unique structural properties. Predominantly, the four intra-molecular hydrogen bonds maintain it by stabilizing the backbone structure. Not least, this is evident from the increase in the number of backbone conformations observed in polar solvents due to the formation of inter-molecular hydrogen bonds with the solvent molecules (Kratochvil et al. 1999). In addition to the four intra-molecular hydrogen bonds, CyA exhibits a cis-amide bond between the N-methylleucine residues at positions 9 and 10. Moreover, the N-methyl moiety of MeVal in the loop makes backbone contacts, which further contribute to the rigidity of the structure (Velkov and Lawen 2003).

2. Biosynthesis

The biosynthesis of bioactive peptides like cyclosporins proceeds non-ribosomally and is catalyzed by complex multi-functional enzymes termed non-ribosomal peptide synthetases (NRPS). Cyclosporin synthetase (CySyn) is one of the best studied enzyme complexes of this type and capable of catalyzing a total of at least 39 different reaction steps in the synthesis of cyclo-undecapaptides via an assembly belt-like mechanism: 11 amino acyladenylation reactions, ten transpeptidations, seven N-methylations, ten chain elongation reactions and a final cyclization reaction (Dittmann et al. 1994; Velkov and Lawen 2003).

The enzyme consists of 11 protein modules, each being responsible for the recognition, activation and modification of one substrate (Lawen and Zocher 1990; Weber et al. 1994) and a small 12th module putatively responsible for cyclization. Based on the gene sequence and the established models for non-ribosomal peptide synthetases (Marahiel et al. 1997), each module of CySyn essentially consists of a central adenylation domain (A-domain;

recognition, activation), a thiolation domain (T-domain, covalent binding of adenylated amino acid on phosphopantethein) and a condensation domain (C-domain; elongation step). During elongation, the activated amino acids are linked by peptide bonds leading to enzyme-bound nascent peptide chains.

CySyn substrates include L-valine, L-leucine, L-alanine, L-glycine, α-amino butyric acid (Abu), 4-methylthreonine, and D-alanine. With the adenulation domain, cyclosporine synthetase generates the acyl-adenylated amino acids and then covalently binds the amino acid to phosphopantetheine through a thioester linkage. Seven of the substrate amino acids become *N*-methylated by *S*-adenosylmethionine via respective methyltransferase activites of CySyn. The final cyclization step releases CyA from the enzyme complex (Hoppert et al. 2001).

Several members of Cy family (like CyA) contain non-proteinogenic amino acids (D-alanine, Abu and unusual Bmt or a similar C9-amino acid; Fig. 8.2), which have to be synthesized by a pathway independent of the primary metabolism. Therefore, besides CySyn, the presence of some other enzymes is crucial for CyA biosynthesis as

well. The biosynthesis of Bmt is catalyzed by a polyketide synthase (PKS) that forms the polyketide backbone by the head-to-tail condensation of four acetate units, resulting in a 3(*R*)-hydroxy-4-(*R*)-methyl-6-(*E*)-octenoic acid thioester; the C-methyl in the carbon chain is derived from AdoMet (Offenzeller et al. 1993). The polyketide 3(*R*)-hydroxy-4-(*R*)-methyl-6-(*E*)-octenoyl-CoA is then transformed into the β-amino acid form which is utilized by CySyn as a substrate for cyclosporine biosynthesis. D-Alanine is provided by a distinct pyridoxal phosphate dependent alanine racemase (Hoffmann et al. 1994). The remaining amino acid constituents of the CyA molecule are synthesized by classic biosynthetic pathways, as confirmed by Senn et al. (1991) using ^{13}C-labeling experiments.

The massive CySyn polypeptide represents the upper limit of molecular size of the NRPS enzymes. A molecular mass of 1.69 MDa (15 281 amino acids), was delineated from the sequence of the CySyn gene, *simA*, which constitutes an intronless genomic open-reading frame (ORF) of 45.8 kb (Weber et al. 1994; Velkov and Lawen 2003). The role of this gene in CyA biosynthesis was proved by Weber and Leitner (1994). They demonstrated that the knock-out of the *simA* gene in *Tolypocladium inflatum* resulted in its inability to produce cyclosporins.
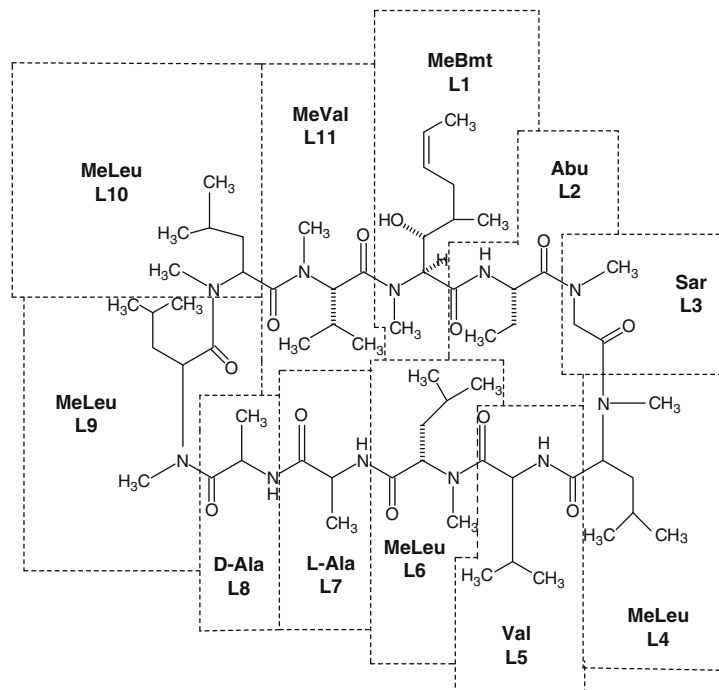

Fig. 8.2. The chemical structure of cyclosporin A including the numbering system. It is composed of 11 amino acid unit, with seven of the amide nitrogen methylated. The three non-proteogenic amino acids are: D-alanine, Abu (L-2 amino-butyric acid) and Bmt (4R)-4-[(E)-2-butyl]-4-methyl-L-threonine (modified from Velkov et al. 2006)

Transmission electron micrographs of negatively stained CySyn macromolecules showed large globular complexes of 25-30 nm in diameter, built up by smaller interconnected units associated with smaller particles of 7 nm length. Complexes of CySyn and D-alanine racemase are linked and localized at the fungal vacular membrane, where Cy synthesis is carried out (Hoppert et al. 2001). CySyn and D-alanine racemase seem to be located in close vicinity to each other, since D-alanine is the leading amino acid of the polypeptide chain synthesized by CySyn. Cyclosporin is subsequently accumulated inside the vacuoles and released slowly through vacuolar and cytoplasmatic membranes or rapidly upon cell lysis.

CySyn was prepared in purified form at pilot scale and used as a model to produce large amounts of CyA in vitro. The process included ammonium sulfate precipitation, gel filtration, hydrophobic interaction chromatography and anion exchange chromatography, and it yielded an electrophoretically homogenous cyclosprin synthetase preparation (Velkov et al. 2006). The obtained enzyme exhibited an optimal temperature range between 24 and 29 ºC and a pH optimum around 7.6.

3. Production

The production of cyclosporins at the laboratory scale can be carried out using different aerobic filamentous fungi such as *Tolypocladium inflatum*, *Fusarium solani* (Sawai et al. 1981), *Neocosmospora vasinfecta* (Nakajima et al. 1989), *Acremonium luzulae* (Moussaïf et al. 1997) and *T. cylindrosporum* (Sekar et al. 1997). The industrial production of cyclosporins is mainly performed using highly productive strains of *T. inflatum*.

This organism was originally mis-classified as *Trichoderma polysporum* Gams, however, later it turned out that it belonged to a new genus of ascomycetous molds, *Tolypocladium* and coined the name *T. inflatum* (Gams 1971). In 1983, another research group found that *T. inflatum* was identical to *Pachybasium niveum*, and since the latter older name has priority under the rules of the International Code of Botanical Nomenclature, the strain was renamed as *T. niveum* (Bissett 1983). This fungus was again re-classified as *Beauveria nivea* (Von Arx 1986). Based on the research of Kathie Hodge, this strain was found to be the asexual state of *Cordyceps subsessilis* (Hodge et al. 1996). Due to the economic importance of this fungus, the classification as *T. inflatum* was nevertheless conserved for cyclosporin producers to avoid any confusion with other strains (Dreyfuss and Gams 1994).

In spite of some efforts to produce CyA with immobilized cells or by solid-state fermentation (SSF), the industrial production of this immuno-suppressive agent is mostly carried out using free cells in submerged cultures in stirred-tank bioreactors. The particular role of the type of strain on the production of CyA along with some characteristic morphological features was reported by several authors.

In case of *T. inflaturm*, large intra-population variations in colony color and shape were observed on solid media. Thus, colony color can range from white to brownish (including yellow, orange and red colonies) (Aarnio and Agathos 1990). The production of a pink pigment was found to be associated with cyclosporin production in certain *T. inflatum* strains (Chun and Agathos 1989). Besides the selection of highly productive colonies of wild-type strains, attempts were undertaken to increase the strain productivity by mutation using chemical mutagens such as methyl sulphate, epichlorohydrin or nitroso-guanidine (Agathos et al. 1986). A recent study of Mi-Jin and coworkers (2009) has demonstrated the possibility of the improvement of *T. niveum* productivity by using random mutagenesis combined with protoplast transformation. The mutant strain, generated using a random UV method, produced more than ninefold higher amounts of CyA than the wild-type strain. Additionally, a bacterial gene of a *Vitreoscilla* spp. (hemoglobin gene, VHb) was transferred to the UV-irradiated mutant to increase oxygen uptake in liquid culture and led to an additional increase in CyA production of more than 30%. Besides the type-strain used, the production levels of CyA are dependent on several regulating factors such as inoculum type and size, medium composition and additives as well as process parameters such as temperature, pH and partial oxygen pressure. A high density of the spore-inoculum was found to be necessary for the development of small pellets, which is the preferred morphology for cyclosporin production (Dayfuss et al. 1976; Isaac et al. 1990). However, inoculum size is only one of more than 20 other factors controlling the fungal pellet formation (El Enshasy 2007).

The influence of the type and concentration of carbon and nitrogen sources on CyA production has been examined in wild-type and mutant strains of *T. inflatum*. Among different carbon sources tested, 3% sorbose gave the highest CyA titre (Agathos et al. 1986). A feeding strategy using the sequential addition of two carbon sources (sorbose and maltose) was also reported to be successful in attaining a higher volumetric production (Agathos et al. 1986). Another study showed that an optimal medium for CyA production can be developed by factorial experimental design and consisted of the three carbon sources glucose, sucrose and starch in different ratios (Abdel Fattah et al. 2007).

Biosynthesis of CyA was found to be heavily influenced by the external addition of amino acid

constituents of the molecule. Addition of L-valine increased the specific production of CyA by 60% in semi-synthetic media and even by 400% in synthetic media. Experiments using repeated addition of L-valine indicated that the amino acid has to be present in the exponential growth phase of the fungus for optimal CyA production (Lee and Agathos 1989). Based on this finding, a mathematical model for the production of CyA in the presence of supplemented L-valine was developed, which also considered kinetic information and mechanistic data on CyA biosynthesis (Agathos and Lee 1993). Concomitant addition of L-leucine and L-valine to a synthetic medium was found to stimulate CyA production as well (Balakrishnan and Pandey 1996). When fungal cells enter the stationary phase and CyA accumulates in the medium, they partially undergo lysis and CyA degradation sets in, especially under carbon source limitation. The intensity of cell lysis and CyA degradation in the bioreactor was higher than in agitated flasks, especially under an uncontrolled pH regime (El Enshasy et al. 2008).

Several attempts have been made to use immobilized cells for CyA production. On example is the successful production of CyA in high amounts using carrageenan-entrapped cells of *T. inflatum* in an airlift bioreactor (Foster et al. 1983). CyA was also produced in relevant amounts by a *Tolypocladium* sp. immobilized in calcium alginate beads in a packed-bed reactor (Sekar and Balaraman 1998a). Continuous production of CyA was realized using immobilized spores of *T. inflatum* on celite beads (Chun and Agathos 1989). The CyA productivity by cells immobilized on celite beads (100–500 μm) was reported to be 4–6 mg l^{-1} h^{-1}. This value is about six- to tenfold higher than those of batch fermentations in suspension cultures (Lee et al. 1997). Furthermore, attempts were made to produce CyA by solid-state fermentation (SSF) to reduce the production costs. So in a study, wheat-bran was used as a solid support and yielded up to 1400 mg CyA kg^{-1} substrate (Sekar et al. 1997). After optimizing different cultivation parameters, such as the type and design of tray, thickness of the solid substrate bed, type and size of inoculum as well as relative humidity, the CyA production increased to a value of 1920 mg kg^{-1} (Sekar and Balaraman 1998b). However, SSF up-scaling raises severe engineering problems due to difficulties of adjusting temperature, pH, oxygen and moisture content as well as of managing gradient formation inside the cultivation system. Recently, a novel process for CyA production by *F. solani* using a large-scale SSF bioreactor of an area of 226 m^2 has been developed (Khedkar et al. 2007). Besides the continuous optimization of the up-stream part of the production process, improvement of CyA extraction methods has also contributed to increase the overall yield of the process. CyA is a hydrophobic molecule with high solubility in low-molecular-weight alcohols, and extraction could be optimized using different alcohols as solvent system and varying temperatures during the extraction process (Ly and Margaritis 2007; Ly et al. 2007).

4. New Generations

A recent study has demonstrated that CyA has a number of side-effects causing among others hypertension, dyslipidemia, hirsuitism and chronic renal insufficiency that leads in 10% of cardiac transplant recipients to an end-stage renal disease (Patel and Kobashigawa 2008). Numerous analogues and derivatives of CyA have been tested in order to improve the drug's therapeutic properties. For example, CyG, a cyclosprin A analogue with a L-novaline substituent at position 2, displays equal immunosuppressive effects as CyA but with less nephrotoxicity (Hiestand et al. 1985). Another derivative SZZ IMM-125, which is a hydroxyethyl derivative of D-serine-8-cyclosporine, was found to be slightly more potent but far less nephrotoxic than CyA in both in vitro and in vivo models (Hiestand et al. 1992; Ferraresso and Kahan 1993). $ISA_{TX}247$ is a potent derivative with higher activity and lower nephrotoxicity compared to CyA (Gregory et al. 2004). Several other cyclosprin analogues with high immunosuppressive activity were obtained through the chemical modification of the side chains at the first amino acid and optionally at third amino acid (Molino and Yang 2006).

B. Mycophenolic Acid

Mycophenolic acid (MPA), [6-(4-hydroxy-6-methoxy-7-methyl-3-oxophthalanyl)-4-methyl-4-hexenic acid; CAS 24280-93-1; Figs. 8.1–8.2], is one of the oldest known secondary metabolites. The compound was first detected in 1896 by Gozio in the fermentation broth of *Penicillium glaucum* and recognized as a lipid-soluble weak organic acid. This compound was also isolated from the culture filtrate of *Penicillium stoloniferum* Thom by Alsberg and Black (1913), who named it MPA (Alsberg and Black 1913; Jekkel et al. 2001). Since then, numerous reports have been published dealing with the production of MPA by different microorganisms. The complete chemical structure of MPA was first reported by Birkinshaw et al (1952).

At the early stage of its biomedical applications, MPA was used as a broad-spectrum antibiotic due to its antibacterial, antifungal, antiviral and antiprotozoal activities (Abraham 1945; Ando et al. 1968; Cline et al. 1969; Noto et al. 1969). Moreover, it was found to exhibit also some antitumor and antipsoriasis as well as anti-inflammatory activities (Carter et al. 1969; Spatz et al. 1978; Epinette et al. 1987). MPA was produced during that time under the trademark Bialin (Vinkurova et al. 2005). However, it has not widely used in practice as antibiotic because most microbes were found to readily become resistant to this compound. In spite of its low acute toxicity to mammals, MPA was also described as a mycotoxin by some authors (Sanchis et al. 1988; Puel et al. 2005). New interest in MPA and its derivatives has remarkably grown after the discovery of their immunosuppressive properties.

The MPA 2-morpholinoethyl ester (also named mycophenolate mofetil, MMF) of the chemical structure 2-morpholinoethyl-(E)-6-(1,3-dihydro-4-hydroxy-6-methoxy-7-methyl-3-oxo-5-isobenzofuranyl)-4-methyl-4-hexeneoate (CAS 128794-94-5; Figs. 8.1–8.3), is one of the most important MFA derivatives and approved by the FDA in 1995 as immunosuppressive agent for the prevention of acute renal allo-graft rejection and in 1998 for heart transplantation. A further improved generation of MPA drugs, based on sodium mycophenolate in controlled release formulations with good gastrointestinal absorption and bioavailability, was approved in 2005 (Xy and Yang 2007). In addition to the well established market for organs transplantations, MPA and its derivatives have been recognized by many physicians as an effective option for the treatment of immune-mediated diseases (Bentley 2000; Mydlarski 2005). Another pro-drug of MPA was developed in Japan during the early 1980s, initially as antitumor agent against various experimental cancers (Mitsui et al. 1981; Matsuzawa and Nakase 1984). In this derivative, the hydroxyl group of MPA was derivatized and the carboxylic functionality was replaced by an ethyl ester to produce ethyl-[N-(p-carboxyphenyl)-carbamoyl]-mycophenolate, abbreviated as CAM. The chemical structure of CAM was fully characterized and its crystal structure solved by Nawata and coworkers (Nawata et al. 1988; 1989). An early study using CAM as immunosuppressive drug demonstrated that it can suppress acute allergic cephalomelitis in Lewis rats (Mizobuchi et al. 1997). Based on this study, it was suggested that CAM might be also a useful adjunct for the long-term immunosuppressive therapy of inflammatory diseases of the central nervous system. At the same time, a study of Sawada and his group demonstrated the usefulness of CAM in bowel transplantation (Sawada et al. 1996). Using a rat model, the immunosuppressive activity of CAM was explained through the inhibition of the interphotoreceptor retinoid-binding protein (IRBP) mediated autoimmune
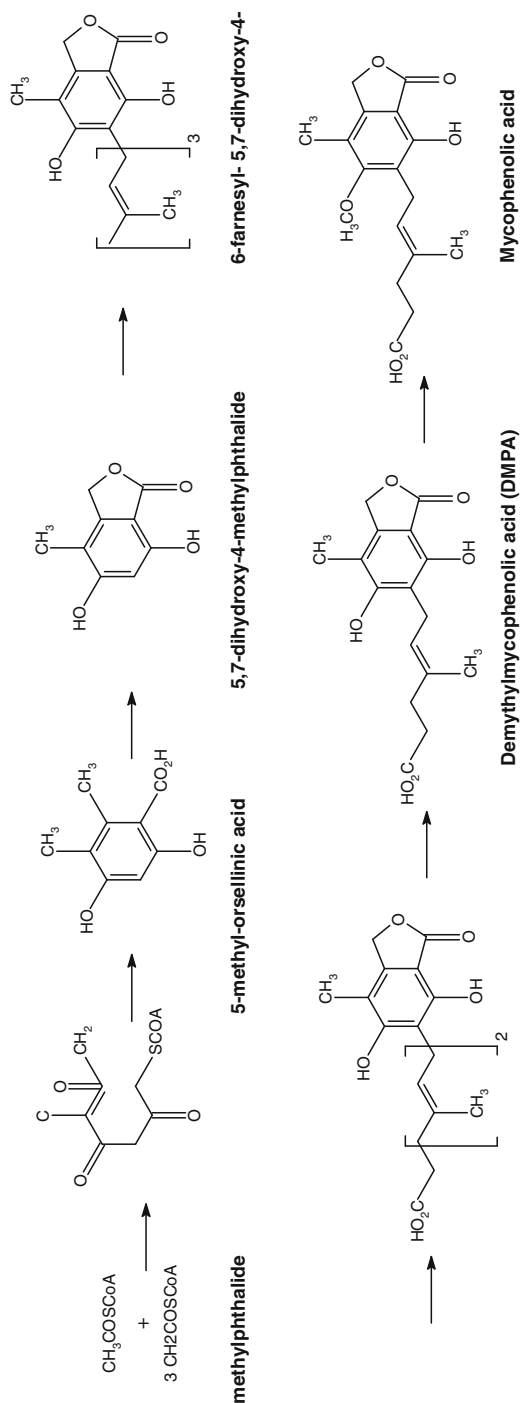

Fig. 8.3. Different steps of MPA biosynthesis

uveoretinitis by a decrease in cytokine production (Sakai et al. 1999). Compared to other immunosuppressive drugs, CAM has only minor adverse side-effects due to its relatively specific action on lymphocytes. Furthermore, this new derivative was more effective than MMF in prolongation of heart-graft survival in rats at each dose applied (Takazawa et al. 1995).

Nowadays, MPA derivatives used as immunosuppressant drug in organ tansplantation are marketed under different trade names, such as CellCept (mycophenolate mofetil, Roche) and Myfortic (mycophenolate sodium; Novartis). In case of oral applications of MMF, the pro-drug mycophenolate mofetil is rapidly hydrolyzed to MPA after administration and suppresses the immune system via a non-competitive reversible inhibition of inosine-5'-monophosphate dehydrogenase (IMPDH; EC 1.1.1.205). This enzyme catalyzes the NAD-dependent oxidation of inosine-5'-monophsphate (IMP) to xanthosine-5'-monophosphate (XMP), which is the committed step in the de novo biosynthesis of guanosine monophosphate (GMP). This reaction is particularly important to generate the guanosine nucleotide levels needed to initiate a proliferation response of B- and T-lymphocytes to mitogens and antigens (Sintchak et al. 1996). Thus, MPA acts as a potent anti-proliferative agent (Hood and Zarembski 1997) affecting cytokine-dependent signals and causing in vivo the inhibition of lymphocyte reactions (Allison and Eugui 2000). In consequence, MPA and its derivatives are being widely used in the transplantation of different organs in humans at different ages (Budde et al. 2006; Tönshoff 2006; Aw et al. 2008).

Besides the wide application of MPA in organ transplantation, it is also used in the treatment of immune related diseases such as rheumatoid arthritis, lupus inflammatory bowel disease and other kidney or skin disorders (Liu and Mackool 2003; Appel et al. 2005; Hartmann and Enk 2005; Iaccarino et al. 2007). Moreover, MPA has recently been used in the treatment of rare diseases like interstitial nephritis (Preddie et al. 2006) and focal segmental glomerulosclerosis (Cattran et al. 2004).

In general, MMF has several advantages over cyclosporins as maintenance therapy of organ-graft recipients (Eugui and Allison 1993). Above all, MMF is well tolerated by the human body and has a lower toxicity and hence fewer side-effects than CyA.

1. Chemistry

Already before the discovery of its immunosuppressive activity, MPA was used as drug due to its wide biological activity against bacteria, parasites and viruses. Thus for many years modifications of the MPA structure were the subject of intensive research in order to increase its biological activity, bioavailability and the range of applications (Lee et al. 1990; Nelson et al. 1990; Rohloff et al. 1995).

Most studies focused on increasing the antitumor activity of MPA through the production of monocyclic analogues and carboxamide derivatives without any change in the aromatic ring and the surrounding side chains, since the free phenolic structure is an absolute prerequisite for MPA activity (McCorkindale and Baxter 1981; Anderson et al. 1996; Menza-Aviñ et al. 2005). The methoxy and methyl groups of the aromatic ring represent two other key structural elements influencing the activity of MPA (El-Araby et al. 2004). The study of Nelson et al. (1996) demonstrated that the aromatic methyl group of MPA is essential for its biological activity and the replacement of the methoxyl group by other ethers resulted in compounds with two- to fourfold higher potency in vitro and in vivo.

Further improvement of MPA activity was achieved by the development of new MPA analogues, which have overcome the drawback of glucuronidation of the phenolic hydroxyl group at C7 (Chen et al. (2007). In these derivatives, a truncated MPA is connected to an adenosine moiety via a linker (e.g. methylene *bis*-phosphonate) leading to mycophenolic adenine dinucleotide derivatives. The new molecules show a better biological activity and chemical stability. Moreover, a new series of IMPDH inhibitors based on the replacement of the benzofuranone moiety in MPA by a methoxy-(5-oxazolyl)-phenyl (MOP) moiety has recently been developed (Chen et al. 2008). Besides the different methods of chemical modification, several attempts were made to transform MPA using different microorganisms (Jekkel et al. 2001). In the course of these bioconversions, mycophenolic acid was found to undergo one or more of the following transformations: hydroxylation at the side chain or the lactone ring, amide or alcohol formation at the carboxylic acid group, oxidative cyclizations of the side chain or glycosylation (Jekkel et al. 2002).

2. Biosynthesis

The molecule of MPA consists of an acetate-derived aromatic nucleus, a terpenoid side chain, and two methyl groups (the 5-methoxyl and the 4-methyl group). Different schemes of MPA biosynthesis have been proposed by different authors (Bedford et al. 1973; Muth and Nash III 1975, Nulton and Campbell 1978). In 2000, Bentley summarized and updated the synthesis pathway in his excellent review (Fig. 8.3). Accordingly, MPA biosynthesis involves two major pathways of secondary metabolite formation: the polyketide and the isoprenoid pathway as well as methylation reactions on oxygen and carbon atoms.

In this process, a typical acetate-polymalonate condensation with a methylation prior to condensation leads to the aromatic structure, 5-methyl-orsellinic acid. It proceeds thereafter through lactone formation followed by addition of the C_{15}-farnesyl diphosphate unit and formation of the intermediate 6-farnesyl-5,7-dihydroxy-4-methylphthalide (Fig. 8.3). This compound undergoes several oxidative degradation steps to remove eight carbon atoms from the side chain by two possible mechanisms: one involves two oxidative cleavages at the two side chain double bonds removing levulinic acid and acetone from the aromatic ring. The other possible mechanism is a direct oxidation at the central double bond of the farnesyl side chain. The two pathways are regarded as being of equal importance (Bentley 2000). Early studies had already shown that the basic carbocyclic skeleton of the molecule was acetate-derived and that methionine provided the O- and C-linked methyl groups attached to the aromatic ring (Birch et al. 1958; Jaureguiberry et al. 1964). The last step of MPA biosynthesis was found to be the transfer of a methyl group from S-adenosyl-L-methionine (SAM) to demethyl-mycophenolic acid (DMPA). This step is catalyzed by a specific SAM:DMP O-methyltransferase (Muth and Nash 1975).

3. Production

MPA was originally isolated from culture filtrates of Penicillium glaucum and P. stoloniferum as a weak acid with antifungal activity (Alsberg and Black 1913); later MPA production was reported for 12 strains of the species P. brevicompactum (Clutterbuck et al. 1932).

Since that time, many reports have been published dealing with the production of MPA using different species of the genus Penicillium (Vinokurova et al. 2005), such as P. brevicompactum (Doerfler et al. 1979; Ozaki et al. 1987a; Alani et al. 2009), P. brunneostoloniferum (Nakajima et al. 1979); P. roqueforti (Lafont et al. 1979, Engel et al. 1982; Schneweis et al. 2000) and other molds like Neocosmospora spp. and Byssochlamys nivea (Puel et al. 2005). Unfortunately, most of the studies on MFA production were carried out on a small scale and data on the detailed effects of media components and cultivation conditions are scarely found in the literature.

Among different MPA producers, the highest productivity was obtained for strains of P. brevicompactum and P. stoloniferum which both are suitable for industrial fermentation (Queener and Nash 1978; Kida et al. 1984; Sircar et al. 2005). Several attempts have been made to improve MPA production by using antibiotic-resistant mutants with a high internal ergosterol level (Queener et al. 1982). Also, rational breeding procedures based on the biosynthetic pathway were used to select strains with improved MPA productivity. Among the different antibiotic-resistant mutants developed, a clofibrate and dodecyltrimehylammonium chloride double resistant mutant produced about 4.7 g l⁻¹ MPA (about three times more than the parent strain, P. brevicompactum ATCC 16024). A glutamate auxotroph of this antibiotic-resistant mutant was even able to produce up to 5.8 g l⁻¹ MPA. This strain was found to grow on L-aspartate instead of L-glutamate and exhibited only one-third of the pyruvate carboxylase activity of the parent strain (Ozaki et al. 1987a).

Cultivation in submerged culture showed that the production of MPA starts concomitantly with the hyphal aggregation phase just before pellet formation (Doerfler et al. 1978). Unlike most secondary metabolites, MPA is produced growth-associated in the exponential phase (in both batch and continuous cultures) and independent of the medium composition (Nulton and Campbell 1977; Doerfler et al. 1979). The production process is carried out either in submerged cultures or by solid-state fermentation (SSF).

For many years, the optimal medium for MPA production has been a semi-synthetic mixture composed of glucose (C-source), ammonium salts or casein (N-source), potassium dihydrogen phosphate (P-source), magnesium sulphate and trace elements. Some authors supplement other components like the amino acid glycine (Xu and Yang 2007) to further increase MPA production. More recently, it has been reported that, among different nitrogen sources, urea in concentrations up to 5 g l⁻¹ was the N-source of choice to support MPA production (Roh 2008). However all in all, only little efforts haven been done to optimize media composition and cultivation conditions compared to the production of other important fungal metabolites.

Like other production processes involving fungal cells, growth morphology is a critical factor determining the growth rate and production yield. Fungi can grow either in form of pellets or mycelia and thus, controlling the growth morphology to a desired shape is important to improve the cell productivity. Altogether, more than 30 factors have been reported in the literature to influence the growth morphology; these include strain-dependent factors (type of strain, inoculums size, physiology, etc.), cultivation conditions (pH, temperature, osmotic stress, etc.) and medium composition (C-source, N-source, C/N ratio, surfactants, presence of insoluble particles) and many other factors (El Enshasy 2007).

Using spores as inoculum for MPA production in submerged culture, it was shown that the increase of spore density from 10^4 to 10^7 spores ml^{-1} resulted in significant reduction in pellet size with a concomitant increase in MPA production from 0.2 up to 4.8 g l^{-1}. Further, it was found that MPA can be continously produced, independent of spore concentration, in the presence of 1% celite in the culture medium. In the case of celite addition, growth occurred in form of pellets (500 μm in diameter) regardless of the inoculated spore concentration (Ozaki et al. 1987b). New cultivation approaches try to overcome the problem of changed cell morphology by applying rotating fibrous-bed bioreactors (RFB). Accordingly immobilized cells in RFBs produced MPA up to a concentration of 5.7 g l^{-1} within 14 days using the standard wild-type strain *P. brevicompactum* ATCC 16024. Other advantages of RFB fermentation include the ease of product separation and purification from the fermentation broth as well as the possible repeated use of cells for long-term operation (Xu and Yang 2007).

Several attempts were made to use solid-state fermentation (SSF) as an alternative cultivation method. In general, filamentous fungi are well suited for SSF and a number of valuable metabolites can be produced under these conditions, since they perfectly reflect the natural habitats of the fungi (Krishna 2005).

An early study by Bartman et al. (1981) demonstrated that, when cells grew as surface culture, MPA production was associated with the aerial mycelium and its production ceased completely when the formation of aerial hyphae was blocked. However, the yield of MPA in this study was relatively low (only 0.3 mg g^{-1} wet weight). SSF production of MPA was optimized using a response surface methodology and the maximal yield achieved was 3300 mg kg^{-1} wheat bran (Sadhukhan et al. 1999). Further improvement was achieved by using a fed-batch strategy (Tiwari et al. 2003). Furthermore, a recent study of Alani et al.

(2009) has demonstrated that MPA production by SSF in a packed-bed bioreactor can lead to yields up to 6900 mg MPA kg^{-1} pearl barley within just 168 h.

Due to its low molecular weight and the relatively simple chemical structure, MPA and its derivatives can nowadays also prepared chemically. Different procedures for MPA total synthesis have been published using different starting materials.

Patterson (1993) synthesized MPA using silyloxy-1,3-cyclohexadiene and allylic alcohol via an *ortho*-ester Claisen rearrangement. Another interesting method for a convergent synthesis of MPA via a palladium-tin coupling reaction between the alkyl side chain and the phthalide ring was described by Plé et al. (1997). A further method involves 2-geranyl-1,3-acetonedicarboxylate and 4-pivaloyoxy-2-butynal and a specific cyclization as key step (Covarrubias-Zúñiga and Gonzlez-Lucas 1998). The production of MPA using this new synthetic strategy is based on a ring annulation sequence involving a Michael addition reaction and an intra-molecular Dieckmann condensation in situ (Covarrubias-Zúñiga et al. 2003).

C. Mizoribine

The immunosuppressive antibiotic mizoribine or bredinin (5-hydroxy-1-β-D-ribofuranosyl-1H-imidazole-4-carboxamide; CAS 50924-49-7; Fig. 8.4) was first isolated from the culture medium of *Eupenicillium brefeldianum* isolated from soil samples on Hachijo island (Japan; Mizuno et al. 1974). Mizoribine (MZA) is an imidazole nucleoside and the metabolite MZ-5-P exerts its activity through selective inhibition of inosine monophosphate synthetase and guanosine monophosphate synthetase, resulting in the complete inhibition of guanine nucleotide biosynthesis (Shumpei 2002). Based on this immunosuppressive mechanism, mizoribine is superior to many
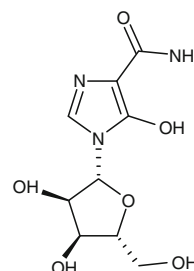

Fig. 8.4. Mizoribine

other clinically used drugs, since it may not cause damage to normal cells and nucleic acids.

In contrast to other immunosuppressive agents widely used at the time of its discovery (e.g. azathioprine), mizoribine was shown in animal experiments to lack oncogenicity and exhibited a clinically low incidence of side-effects such as hepatotoxicity and myelosuppression. These facts together supported its use in clinical application in long-term immunosuppression therapies. In 1984, MZR was first approved for the treatment of graft rejection after kidney transplantation (Takei 2002). Later, it was also approved for the treatment of other diseases including lupus nephritis, rheumatoid arthritis and primary nephritic syndrome. It is currently marketed in China, Korea and Japan under the trade name Bredinin (Tanaka et al. 2006). The drug is mainly produced by the fungi *E. brefeldianum* and *E. javanicum* in submerged culture under aerobic conditions, however, little information is available on the biosynthesis pathway and the production process (Mizuno et al. 1975; Benedetti et al. 2002).

D. Other Immunosuppressants

In addition to the three clinically approved fungal immunosuppressants, Cyclosporins, MPA and mizoribine, many other fungal metabolites possess also potent immunosuppressive activities (Fig. 8.5). Some of them were found to be not suitable for clinical applications due to their side-effects, whereas others are still subject of intensive studies and currently undergoing different levels of clinical trials till final approval by FDA.

1. Ovalicins and Fumagillins

The fungal metabolites ovalicin, fumagillin and their related derivatives belong to the most potent anti-angiogenic compounds. They bind covalently to the active site of the enzyme methionine-aminopeptidase type 2 (MetAP2) and irreversibly block its proteolytic activity (Liu et al. 1998; Turk et al. 1998).

Ovalicin or Graphinone (CAS 19683-98-8) is a sesquiterpene that was first isolated from culture filtrates of *Pseudeurotium ovalis* in 1962 and found to have antimicrobial and cytotoxic activities. It was chemically characterized by Sigg and Weber (1968). The chemical structure of this compound is related to the antibiotic fumagillin. The immunosuppressive properties of ovalicin were evaluated using the mouse hemagglutinin test, which reflects the degree of antibody production. It was found be not toxic to the cells of bone marrow, which distinguished it from existing immunosuppressants at that time. Unfortunately, when tested in humans it later turned out to have other toxic side-effects.

A *Metarhizium* sp. isolated from soil in Japan was found to produce a 12-hydroxyovalicin (Kuboki et al. 1999), which was named Mer-f3. This compound was examined for its influence on mixed lymphocyte cultures, and showed a similar inhibitory activity as ovalicin. Mer-f3 had an immunosuppressant activity in the murine mixed lymphocyte test with $IC_{50} = 1$ nM which is even better than that of CyA (110 nM). Moreover, Mer-f3 had no inhibitory activity on leukemia L-1210 which indicates a low mammalian toxicity. Another *Metarhizium* sp. isolated from soil was reported to produce a novel immunosuppressive substance, which was named metacytofilin (Iijima et al. 1992); this compound had not any antimicrobial activity, while showing strong immunosuppressive effects.

Chlovalicin, a chlorinated compound derived from the epoxide ring attached to ovalicin, was discovered in the fermentation broth of the soil fungus *Sporothrix* sp. It inhibited the IL-6 dependent growth of MH60 cells and appeared to be a new IL-6 inhibitor (Hayashi et al. 1996). Two ovalicin related compounds, FR 65814 (CAS 103470-60-6) and Fumagillol (CAS 108102-51-8), were isolated from culture filtrates of the soil fungus *Penicillium jensenii*. They both showed significant immunosuppressive activity at low concentrations (Hatanaka et al. 1988). A chiral and stereoselective total synthesis of FR 65814 using glucose as starting material was described by Amano et al. (1998, 1999), and fumagillol can be also synthesized using other starting materials (Kim et al. 1997, 2005a; Boiteau et al. 2001). TNP-470, a semisynthetic derivative of fumagillin, reduced the proliferation of endothelial cells with an IC_{50} value of 2.5×10^{-11} M. Therefore, it entered clinical trials as immunosuppressive and anti-tumor agent. The main drawbacks of its therapeutic properties were the short physiological half-life span and the severe side-effects such as ataxia, vertigo and agitation (Figg et al. 1997). The relation between the chemical structure and bioactivity of fumagillin and its derivatives was studied by several authors. It was shown that the spiro-epoxide is essential for the activity of fumagillin and its conversion into a methylene group results in a considerable reduction of eficacy (Logothetis et al. 2001). On the other hand, the epoxide of the side chain has no major effect on the biological activity. New synthetic analogues of ovalicin and fumagillin lacking reactive epoxy functionalities, which are thought to be responsible for the severe toxic side-effects, were synthesized by Mazitschek et al. (2005).

2. Gliotoxin

Gliotoxin (CAS 67-99-2) is a sulfur-containing antibiotic that belongs to the epipolythiodioxopiperazine group of secondary metabolites and
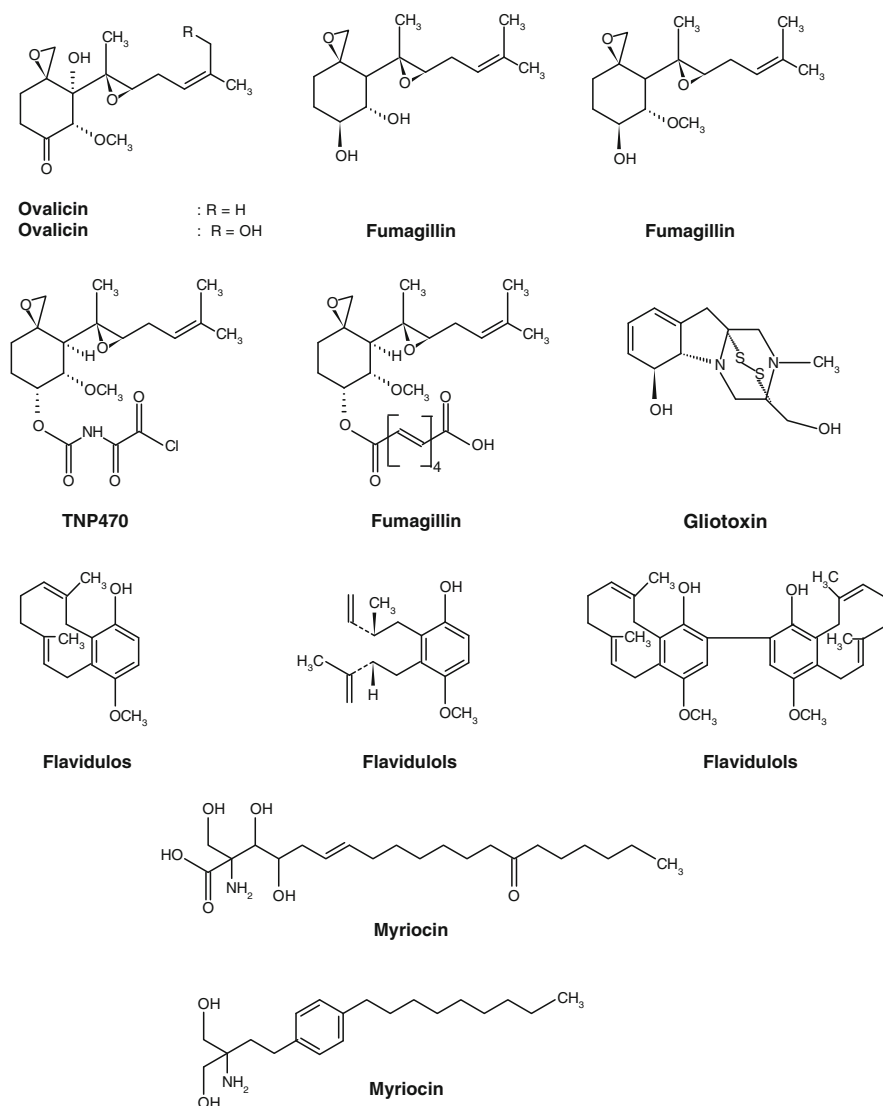
Fig. 8.5. Molecular structure of different types of fungal immunosuppressive agents

exhibits antifungal, antiviral as well as strong immunosuppressive activities. Gliotoxin was originally isolated from *Gliocladium fimbriatum* and named accordingly. It was reported thereafter that this compound is commonly produced by several genera of molds such as *Aspergillus*, *Trichoderma* and *Penicillium*. Gliotoxin was also claimed to be produced in yeasts of the genus *Candida*, however, a recent study being based on a screening of 100 clinical isolates of *Candida* doubted the occurrence of this compound in yeasts (Kupfahl et al. 2007).

The immunosuppressive effects of gliotoxin have been explained by different mechanisms. It suppresses cell activity and induces apoptosis in a variety of cell types including neutrophils, esosinophils and granulocytes (Ward et al. 1999). It inhibits the chymotrypsin-like activity of the 20S proteasome in a non-competitive manner (Kroll et al. 1999) and likewise the activation of NF-*k*B in T- and B-cells when applied at nanomolar concentrations (Pahl et al. 1996). The immunosuppressive activity of gliotoxin was also attributed to the inhibition of perforin-dependent and Fas-ligand-dependent cytotoxic T-lymphocyte-(CTL)-mediated cytotoxicity (Yamada et al. 2000). Another study has furthermore demonstrated that gliotoxin suppresses the mast cells, which play a key
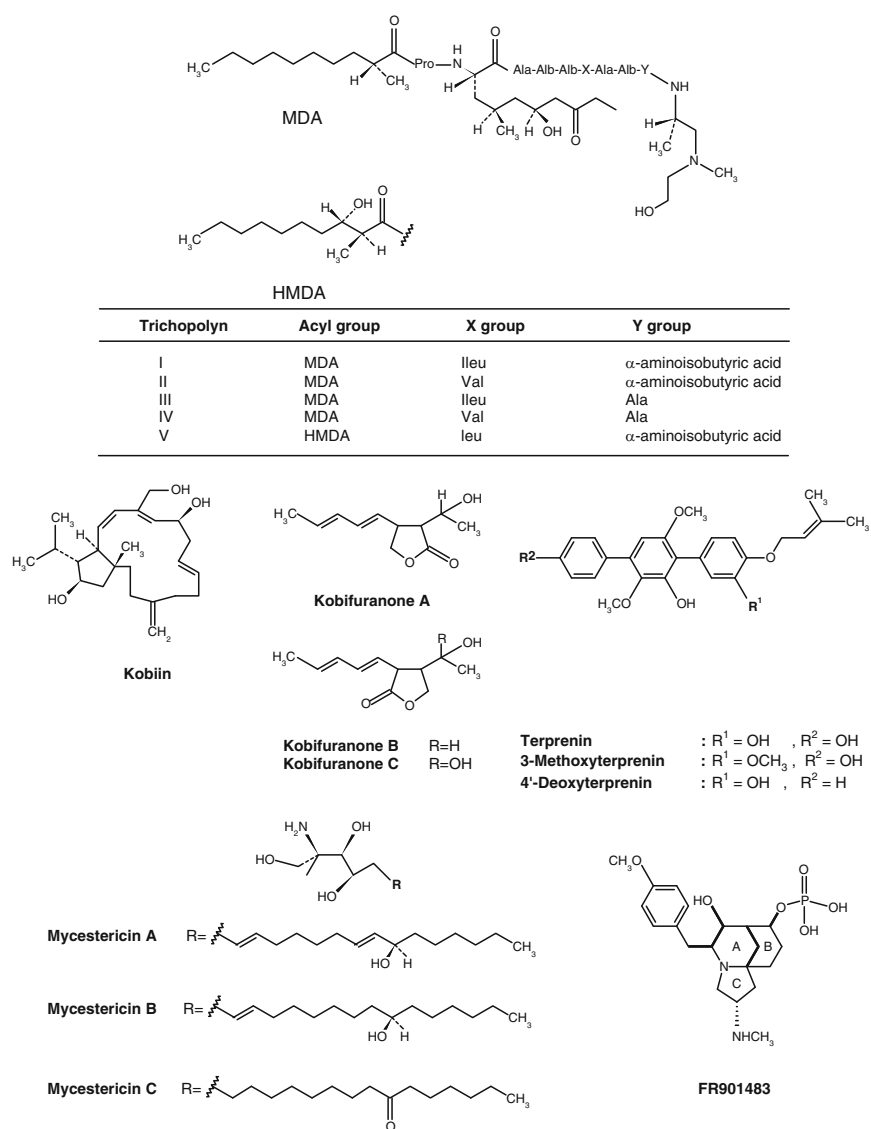
Trichopolyn	Acyl group	X group	Y group
I	MDA	Ileu	α-aminoisobutyric acid
II	MDA	Val	α-aminoisobutyric acid
III	MDA	Ileu	Ala
IV	MDA	Val	Ala
V	HMDA	Ieu	α-aminoisobutyric acid

Fig. 8.5. (continued)

role in host defense and are important in both innate and adaptive immunity (Niido et al. 2006). As this compound is produced by many potential human pathogens in vivo during the course of infection, gliotoxin may also contribute to the etiology of fungal diseases (Waring and Beaver 1996).

3. Trichopolyns

Trichopolyns (TPs) are peptabiotic compounds produced by *Trichoderma polysporum*. TPs I and II were first isolated as new antifungal and antibacterial antibiotics in 1978 by Fuji and coworkers. The chemical structures of TP I (CAS 66554-87-8) and TP II (CAS 6655-88-9) were identified as peptide antibiotics three years after their discovery (Fujita et al. 1981). Nowadays, the structures of five different trichopolyns (I–V) are known. Trychopolyns I and II are ten-residue peptides characterized by the presence of a 2-methyldecanoyl group at the N-terminus, and the C-terminal residue is protected by trichodiaminol. The other three analogues, TPs III-V differ from TPs I and II in that way that Aib (α-aminoisobutyric acid) is replaced by L-alanine. In contrast, TP V has the same amino acid sequence as

TP I, but the N-terminal acyl group is substituted by 3-hydroxy-2-methyldecanoic acid (instead of 2-methyldecanoic acid). These peptabiotics have been shown to suppress the proliferation of lymphocytes in mouse allogeneic mixed lymphocyte reactions (Lida et al. 1999). The TP I activity was even stronger than that of CyA.

4. Myriocin

Myriocin (antibiotic ISP-1 or thermozymocidin; CAS 35891-70-4) was first isolated from the thermopholic fungus *Myriococcum albomyces* by Kluepfel and his group in 1972 and patented in the United States in 1975 (Kluepfel et al. 1975). The compound was recognized as an active antibiotic against yeasts and dermatophytes when applied in vitro. However, the compound appeared to be too toxic for therapeutic purposes in humans.

More than 20 years later, the same compound and its derivatives (mycestericins) were isolated from *Isaria sinclairii*, which is the imperfect stage of *Cordyceps sinclairii*, and showed strong immunosuppressive activities. Myriocin was 10- to 100-fold more effective than cyclosporin A both in in vivo and in vitro tests (Fujita et al. 1994; Sasaki et al. 1994). *Isaria* sp. belong to the entomopathogenic fungi colloquially called "vegetable wasps and plant worms", which have been used in oriental medicine for more than 1000 years (Im 2003). Myriocin was found to suppress both the production of antibodies against red blood cells of sheep and the induction of cytotoxic T-lymphocytes more strongly than cyclosporine A. It is also a potent inhibitor of serine palmitoyltransferase (SPT), the enzyme that catalyzes the first step in sphingosine biosynthesis (Miyake et al. 1995). Thus, it is used in biochemical research as a tool for depleting cells of sphingolipids. Myriocin can also be produced by chemical methods (Banfi et al. 1982; Oishi et al. 2002; Jones and Marsden 2008).

Fingolimod or FTY720 (CAS 162359-55-9) is a novel immunosuppressant obtained by chemical modification of myriocin (Adachi et al. 1995). A number of alternative ways for its preparation using shorter pathways for synthesis with higher overall yields have been published over recent years (Seidel et al. 2004; Adachi and Chiba 2007). Fingolimod was actually designed to eliminate the GI toxicity of the original compound myriocin. The exact mechanism of its immunosuppressive activity, however, remains still unclear. Some researchers have hypothesized that FTY720 may induce the apoptosis of lymphocytes (Suzuki 1996; Fujino et al. 2002). Others have proposed that the number

of lymphocytes decreases as a result of their movement towards secondary lymphoid organs such as lymph nodes and peyer's patches (Sugito et al. 2005). It is certain that this novel immunosuppressant prolongs the survival of allo-graft transplants and is effective in the treatment of some immunological diseases. At the moment, FTY720 is being further developed by Novartis in phase II clinical trials. Not least, this compound may have a great clinical potential because of eficacy as oral drug for the treatment of multiple sclerosis (Gullo et al. 2006; Klatt et al. 2007).

5. Flavidulols

The immunosuppressive geranylphenols, flavidulols A (CAS 117568-32-8), B (CAS 117568-33-9) and C (CAS 117568-34-0), were originally isolated from fruiting body extracts of the mushroom *Lactarius flavidulus* in the course of a screening for new inhibitors of the proliferation of mouse lymphocytes (Takahashi et al. 1988). The chemical structure of these compounds was determined by NMR analysis (Takahashi et al. 1993); this paper also reported on the isolation of a new flavidulol D (CAS 156980-40-4). The suppressive effects of flavidulols A, B and C on the proliferartion of mouse lymphocytes were stimulated in the presence of mitogens such as concavalin A (CoA) and lipopolysaccharides (LPS). Their IC_{50} values for the inhibition of mitogen-induced concavalin A proliferation of mouse lymphocytes were between 9 and 36 µg ml^{-1} and against lipopolysaccharide-induced proliferation between 7 and 28 µg ml^{-1} (Fujimoto et al. 1993).

6. Kobiins

The sesterterpenetriol immunosuppressant kobiin and another three related 2-furanones named kobifuranones A, B and C were first isolated from the ascomycetes *Gelasinospora kobi* by Fujimoto et al. (1998). Kobiin posses a bicyclic skeleton of five- and fifteen-membered rings. Kobifuranones A, B and C were supposed to be metabolites formed from a common intermediate biosynthesized through the acetate-malonate pathway. AcOEt extracts of fungal mycelia containing kobiin and the three kobifuranones were found to suppress proliferation of mouse spleen lymphocytes stimulated with the mitogens CoA

and LPS. After solvent fractionation followed by repeated chromatography, the purified kobiin prepartion obtained showed the highest immunosuppressive activity.

7. Mycestericins

Mycestericins are a group of unique immunosuppressive compounds and chemically, hydroxylated α-hydroxymethyl α-aminoalkanoic acids. All known types of mycestericins were isolated from the cultures of *mycelia sterila* (i.e. filamentous fungi without any morphological structures, neither sexual organs and spores nor conidia and other asexual spores). The chemical structures of mycestericin A (CAS 128440-98-2), B (CAS 128341-87-7), C (CAS 37817-99-5), D (CAS 157183-67-0) and E were determined on the basis of comprehensive spectroscopic studies and chemical tests (Sasaki et al. 1994). Mycestericins suppress the proliferation of lymphocytes in the mouse allogeneic mixed lymphocyte reaction with a potency similar to that of myriocin. Further studies led to the isolation of two more active compounds, mycestericins F and G, from the same fungus (Fujita et al. 1996). The chemical structures of mycestericins F and G were identical to the respective dihydromycestericins D and E. Mycestericin A has also been chemically synthesized using simple tartrate as starting compound (Sato et al. 2008); total chemical synthesis of mycestericins E was accomplished by a cinchona alkaloid-catalyzed asymmetric Baylis-Hillman reaction (Iwabuchi et al. 2001). Mycestericins D–G can enzymatically be prepared using L-threonine aldolase from *Candida humicola* in the key step reaction (Nishide et al. 2000).

8. Terprenins

Terprenin (CAS 197899-11-9) was discovered by Kamigauchi et al. (1998) in the fermentation broth of *Aspergillus candidus* during a screening for natural immunosuppressants (Kamigauchi et al. 1998). It has a novel highly oxygenated *p*-terphenul structure with a prenyloxy side chain. Two terprenin derivatives, 3-methoxy-terprenin and 4′-deoxyterprenin, were also isolated from the fermentation liquid and showed significant immunosuppressive effects when tested with respect to the proliferation of mouse spleen lymphocytes.

The most relevant activity of terprenin is its suppressive effect on the production of immunoglobulin E (IgE), which is a factor of 10^4 stronger than that of FK506, and interestingly, without any toxicological side-effect (Kawada et al. 1998). In mice experiments, terprenin suppressed IgE production in a typical dose dependent manner. Even after immunization with ovalbumin, when the IgE value had reached a high level, terprenin still exhibited a significant suppressive effect at 20–40 mg kg^{-1} (Liu 2006). The total synthesis of terprenin is possible and was reported by different authors (Kawada et al. 1998; Yonezawa et al. 1998).

9. FR901483

A potent immunosuppressant, FR901483, was isolated in 1996 from the fermentation broth of *Cladobotryum* sp. by Fujisawa Pharmaceutical Co. in Japan (Sakamoto et al. 1996). It was found to exert a potent immunosuppressive activity in vitro and significantly prolonged graft survival in the rat-skin allograft model, apparently by the inhibition of purine nucleotide biosynthesis. This compound has an intriguing tricyclic structure possessing a phosphate ester in its molecule. Since its discovery, FR901483 has garnered significant attention from the organic chemists due to its biological activity and unique aza-tricyclic nature. Thus, different synthesis schemes were published for the total synthesis of this important immunosuppressant (Maeng and Funk 2001; Kropf et al. 2006; Carson and Kerr 2009).

10. Colutellin A

Colutellin A is a new immunosuppressive peptide recently isolated from *Colletrichum dematium*. It showed CD$^+$ T-cell activation of interleukin 2 (IL-2) production with an IC$_{50}$ of 167 nM. Moreover, it exhibited no cytotoxicity to human peripheral blood mononuclear cells in respective in vitro tests. Thus, it could be medicinally used as a novel immunosuppressive compound in the near future (Ren et al. 2008).

III. Mushroom Immunomodulators

Nowadays, immunostimulators (biological response modifiers) are becoming increasingly more popular in the health and wellness industries

as people have started to realize the importance of a healthy immune system as a first barrier for the prevention of diseases. These pro-drugs or pro-phylactic medicines have a long history in traditional medicine, in particular in Asian and Mediterranean countries. Thus the medical use of mushrooms has a long tradition in Japan, China, Korea and Southeast Asia, whereas, in Europe and the United States, this field has just been developing since the early 1980s. The positive medicinal properties of mushrooms are based on various cellular compounds and secondary metabolites, which can be isolated from different parts of the fruiting body or from the mycelium during growth in solid-state or liquid cultures (Tang et al. 2007). The immunomodulating effects of mushroom metabolites are especially valuable in the prophylaxis as a mild and non-invasive form of a treatment, which can even prevent the proliferation of metastatic tumors, and is used as a co-treatment in combination with classic chemo- and radiotherapies (Wasser 2002). The most potent immunomodulators produced by mushrooms belong to the lectins, terpenoids and polysaccharides.

A. Lectins

The immunomodulatory activities of lectins (highly glycolylated proteins with specific binding capacities) from different organisms have been known for decades. Mushroom lectins are characterized by their particular antiproliferative and antitumor activities.

Boletus satanas lectin, bolesatine, was shown to have a potent mitogenic activity on human peripheral blood lymphocytes, and also to stimulate the release of IL-1α, IL2 and TNF-α from mononuclear cell cultures (Licastro et al. 1993). A fruiting- body lectin of *Grifola frondosa* showed cytotoxic activity against HeLa cells, when applied at low concentration (Kawagishi et al. 1990). A heterodimeric melibiose-binding lectin from fruiting bodies of the oyster mushroom *Pleurotus ostreatus* was reported to be an in vivo inhibitor of sarcoma S-180 and hepatoma H-22 tumor cells (Wang et al. 2000). A specific lectin was identified in fruiting bodies and mycelia of the straw mushroom (*Volvariella volvacea*) and had a stronger immunomodulatory effect than concanavalin A (She et al. 1998). Two lectins, TML-1 and TML-2, with immunomodulatory and antitumor activities were isolated from *Tricholoma mongolicum*; however, when these lectins were directly tested in vitro, no antitumor activity was observed. This suggests that the lectins are rather immunomodulatory substances than substances exerting acute cytotoxicity. Peritoneal macrophages in mice treated with TML-1 or TML-2 revealed – after LPS stimulation – an enhanced production of nitrite and TNF-α. Both compounds inhibited also the growth of P815 mastocytoma cells by stimulating peritoneal macrophages to produce more macrophage-activating factors including interferon-γ and some other cytokines (Wang et al. 1996, 1997).

B. Terpenoids

Terpenes are built up of isoprene sub-units consisting of five carbon atoms. Among the huge number of terpenes, are special triterpenoids which are exclusively found in certain macrofungi (mostly basidiomycetes) and are famous for their biological activities and medicinal properties. One example of such a triterpenoid compound is the highly oxidized lanostane which can be isolated from wood-decay fungi of the families Polyporaceae and Ganodermaceae (e.g. *Ganoderma lucidum*). This and related compounds show different biological activities including anti-infective, cytotoxic and immunomodulating efficacy (Moradali et al. 2007). Ganoderic, ganoderenic, ganodermic and applanoxidic acids, ganoderals, ganoderols, lucidone, ganodermanontriol as well as ganodermanondiol are the most common triterpenoids found in these mushrooms. Mixtures of these compounds can be prepared by the extraction of respective fruiting bodies with organic solvents. They were shown to have an antitumor activity that is comparable to that of certain β-D-glucans (see below). Fungal terpenoids can stimulate the NF-kB pathway and modulate Ras/Erk, c-myc and CREB proteins as well as mitogen-activated protein kinases (Gao et al. 2003). In consequence, these activation mechanisms can lead to other immune stimulations which are finally effective against tumor cells.

C. Polysaccharides

Various polysaccharides of microbial and non-microbial origin have been widely used as potential biological response modifiers (BRMs) as reviewed by Leung et al. (2006). Mushroom derived polysaccharides are regarded as excellent

immunostimulators due to their suitable therapeutic properties, i.e. they are barely toxic and have just negligible side-effects compared to other immunostimulants. Respective polysaccharides occur in relevant amounts in the macroscopic fruiting bodies and cultured mycelia but also to some extent in the culture filtrates of fungi. Most macrofungal polysaccharides belong either to the homoglycans or heteroglycans, and can bind to structure proteins to form polysaccharide-protein complexes. In general, immunomodulator polysaccharides appear to be related to the fungal cell wall and comprise (1→3)- and (1→6)-β-glucans as well as (1→3)-α-glucans or polysaccharide complexes of the galactomannan- and glucuromannan-protein type.

Research on mushroom polysaccharides can be traced back to the 1960s when Ikegawa's group in Japan first investigated the host-mediated antitumor activity of hot-water extracts of several edible mushrooms against sarcoma 180 cells of mice (Ikekawa et al. 1969). Until the late 1980s, three antitumor-immunomodulators of the β-glucan type were isolated and characterized, namely lentinan, schizophyllan and a protein-bound β-glucan (PSK Kresin). They originate from the white-rot fungi *Lentinus edodes*, *Schizophyllum commune* and *Coriolus versicolor*, respectively, and have successfully been introduced into the probiotic and pharmaceutical market in Japan. A similar polysaccharopeptide as PSK, abbreviated as PSP is produced in China and widely used in the clinical treatment of tumors and in anticancer therapy (Ooi and Liu 2000). Although their mode of action against tumore cells is not yet fully understood, they have been demonstrated to act as biological response modifiers (BRMs), which are able to restore or enhance various immune responses in vitro and in vivo.

The mushroom polysaccharides or polysaccharide-protein complexes stimulate the non-specific immune system and thereby exert antitumor activities through the stimulation of the body's own defence mechanisms (Wasser and Weis 1999; Reshetnikov et al. 2001). They can activate effector cells like macrophages and T-lymphocytes or prompt NK cells to secrete cytokines like TNF-α, IFN-γ and IL-1β. In turn, some of these cytokines are able to directly promote the cytotoxicity of macrophages. The production of cytokines by immune cells can be considered as a key event in the initiation and regulation of the body's immune response (Lull et al. 2005). In this context, mushroom polysaccharides can act as antiproliferative effectors and induce apoptosis in tumor cells.

Certain mushroom polysaccharides (e.g. BRMs) were shown to reduce the tumor size by more than 50% and considerably prolonged the survival of tumor-bearing mice (Wasser 2002). Though the exact mechanism of BRM action is not known, it has been proposed that they initiate a cascade of singal transduction that is responsible for the immune response. Since polysaccharides are not able to penetrate cells (due to their high molecular mass), the first step of the cascade may be the recognition of BRM and its specific binding to immunocell receptors. Some evidence exists that there are pattern recognition receptors (PRRs) for the molecular reception of the polysaccharide BRM (Lowe et al. 2001). The binding of the BRM-ligand to PRRs may initiate Rel/NF-*k*B-mediated signaling events, which leads to the induction of gene expression and the stimulation of specific cellular functions of the innate immunity system (Leung et al. 2006). Whilst it is known that mushroom extracts have immunomodulatory activity, the standard approach has always been to isolate, characterize and administer pure active compounds. However, different types of polysaccharides in a mushroom extract may have synergistic acitivities (Borchers et al. 2004; Lull et al. 2005). The responses to different polysaccharides are mediated by different PRRs on the cell surface. An appropriate combination of strong responses involving different parts of the cell may provide greater therapeutic effects than a single polysaccharide. A brief list of immunomodulator polysaccharides and polysaccharide–protein complexes from mushroom is given in Table 8.1.

Mushroom polysaccharides greatly differ in their sugar composition, branching configuration, helical conformation and other physical properties. The structure relationship between immunomodulator and the anticancer activities of polysaccharides have been reviewed by several authors (Ooi and Liu 2000; Lull et al. 2005; Zhang et al. 2007; Ooi 2008). It has been stated that structural features such as (1→3)-β-linkages in the main chain of the glucan and additional (1→6)-β-branching points, represent important factors influencing their biological effectiveness. β-Glucans containing mainly (1→6)-linkages are less effective, maybe due to their inherent flexibility and the large number of possible conformations (Zhang et al. 2007). In general, β-glucans exhibit immunomodulatory and/or antitumor activities when their main chain ("backbone") forms a linear structure and do not have long branches. For example, pachyman, a branched (1→3)-β-D-glucan obtained from the brown-rot fungus *Poria cocos* is inactive, whereas pachymaran obtained by the debranching of pachyman using selective periodate oxidation and mild hydrolysis, shows a pronounced activity (Chihara et al. 1970a).

Lentinan (2/5) and schizophyllan (1/3) are (1→3)-β-D glucans with two or one branches for every 5-D-glucopyranosyl and 3-D-glucopyranosyl residue, respectively (Tabata et al. 1981; Chihara 1992). The polysaccharide moiety in PSK (1/5) is a (1→3)-β-/(1→4)-β-D-glucan with one branch for every 5-D-glucopyranosyl residue (Tsukagoshi et al. 1984). Although the degree of their branches is different, their bioactivities are similar (Ooi and Liu 2000). Interstingly, debranched lentinan preparations were found to be more effective against cancer cells than the native lentinan when applied during in vivo studies

Table 8.1. Immunomodulator polysaccharides from mushrooms

Mushroom species	Polysaccharide source	Active component	References
Coriolus versicolor	Mycelium, Culture broth	Polysaccharopeptide	Cui and Chisti 2003
Cordyceps sinensis.	Fruiting body, Mycelium, Culture broth	Glucan, heteroglycan, Cordyglucan	Yalin et al. 2005 Russell and Paterson 2008
Flammulina velutipes	Fruiting body, Mycelium	Glucan-protein complex, glycoprotein	Leung et al. 1997
Ganoderma lucidum	Fruit body, Culture broth	Heteroglycan, mannoglucan, glycopeptide	Miyazaki and Nishijima 1981 Gao and Zhou 2003
Grifola frondosa	Fruiting body, Culture broth	Proteoglycan, glucan, heteroglycan, galactomannan, grifolan	Cun et al. 1994 Yang et al. 2007
Hericum erinaceus	Fruiting body, Mycelium	Heteroglycan, Heteroglycan-peptide	Mizuno 1992
Inonotus obliquus	Fruiting body, Mycelium	Glucan	Kim et al. 2005b
Lentinus edodes	Fruiting body, Culture broth	Mannoglucan, glucan, Lentinan, polysaccharide protein complex	Chihara et al. 1970b Hobbs 2000
Peziza verculosa	Fruiting body	Glucan, Proteoglycan	Mimura et al. 1985
Pleurotus ostreatus	Fruit body, Mycelium, Culture broth	Heteroglactan, Proteoglycan	Sarangi et al. 2006
Polyporus umbellatus	Mycelium	Glucan	Yang et al. 2004
Polystrictus versicolar	Fruiting body, Mycelium, Culture broth	Heteroglycan, glycopeptides, krestin (PSK)	Cui and Chisti 2003
Sclerotinia sclerotiorum	Sclerotium	Glucan, scleroglucan (SSG)	Palleschi et al. 2005 Survase et al. 2007
Tremella aurantialba	Fruiting body	Heteroglycan	Liu et al. 2003
Tricholoma lobayense	Culture broth	Polysaccharide-protein complex	Liu et al. 1996

(Sasaki et al. 1976). Overall, the relationship between the molecule's biological activity and the branching pattern/ratio of β-glucans seems to be rather complicate. The available data indicate that the (1→3)-β-D-glucan backbone is essential and that the most active polymers have degrees of branching between 0.2 and 0.33 (Ooi 2008). The molecular weight of the polysaccharide plays also an important role for the bioactivity. For a (1→3)-β-glucan extract of *G. frondosa* consisting of fractions of different size, the highest immunomodulatory activity was detected for molecular masses around 800 kDa (Adachi et al. 1990). When PSK was separated into four fractions (F1, <50 kDa; F2, 50–100 kDa; F3, 100–200 kDa, F4, >200 kDa) by successive ultrafiltration, the highest immunomodulatory activity was obtained with the high-molecular mass fraction F4 (Kim et al. 1990). Chemically modified (1→3)-β-D-glucans, such as schizophyllan and lentinan having a linear "worm-like", triple-helical structure and average molecular masses of <50 000 g mol^{-1} or >110 000 g mol^{-1} efficiently stimulated monocytes in vivo and caused the secretion of more TNF-α than the samples with molecular masses between 67 000 and 110 000 g mol^{-1} did Therefore, the actual

relation between the polysaccharide molecular mass and its immunomodulation activity remains to be clarified. Conformations of polysaccharides include single helices and triple-helices as well as random-coiled structures. A triple-helix conformation is usually more stable than a single-helix. Lentinan, schizophyllan and the glucan moiety of PSK have all triple helix structures. Also the cytokine-stimulating activity of (1→3)-β-D-glucans was found to be associated with the triple-helix conformation (Falch et al. 2000). Therefore, the immunological activities of polysaccharides must be dependent on appropriate helical conformation.

To improve the biological activity of polysaccharides by chemical modification, carboxymethylated, hydroxylated, formylmethylated, aminethylated and sulfated products have been designed. For example, a hydroxylated schizophyllan was found to induce in vivo the production of higher concentrations of nitric oxide (NO) and TNF-α in macrophages than native

schizophyllan (Ohno et al. 1995). A sulfated (1→3)-α-D-glucan prepared by respective modification of native cell-wall glucan from *L. edodes* exhibited a strong antiproliferation activity against breast carcinoma cells, whereas its native water-insoluble precursor glucan had only moderate antitumor activity (Zhang and Cheung, 2002). Another study conducted by Zhang et al. (2004) showed that the high bioactivity of carboxymethylated β-glucan was mainly attributed to the increase of water-solubility and the latter was also the main factor that enhanced the efficacy of the hyperbranched β-glucan TM3b after sulfatation (Tao et al. 2006).

D. Fungal Immunomodulator Proteins

Recently, different mushrooms have been reported to produce a new family of fungal immunomodulatory proteins (FIPs) with possible applications in therapy (Chen and Wang 2007). These include Ling Zhi-8 (LZ-8) from *Ganoderma lucidum* (Kino et al. 1989), FIP-fve from *Flammulina velutipes* (Gr.) Sing (Ko et al. 1995), FIP-vvo and FIP-vvl from *Volvariella volvacea* (Bull.; Fr.) sing (Hsu et al. 1997), FIP-gts from *Ganoderma tsugae* Murr (Lin et al. 1997) and PCP from *Poria cocos* (Schw.) Wolf (Chang and Sheu 2007). All these compounds were grouped together in a distinct protein family based on similarities in their amino acids sequence and their effects on compounds of the immunological response system (Ko et al. 1995).

FIPs were found to be mitogenic in vitro for human peripheral blood lymphocytes (hPBLs) and mouse splenocytes. They induce a bell-shaped dose–response curve similar to that of lectin mitogens. In the course of in vivo studies, the FIP-like substance LZ-8 could act as an immunosuppressive agent through the prevention of systemic anaphylactic reactions and significantly decreased footpad edema during the Arthus reaction (Tanaka et al. 1989). Moreover, it suppressed autoimmune diabetic reactions in diabetic mice and increased graft survival in transplanted allogenic mouse skin and pancreatic rats without producing the severe toxic effects known for CyA (Van der Hem et al. 1994; 1996). FIP-fve isolated from fruiting bodies of *F. velutipes* stimulated mitogenesis of human peripheral lymphocytes, suppressed systemic anaphylaxis reactions and enhanced the transcription of interleukin-2 (IL-2) and interferon-γ (Ko et al. 1995). The induction mechanism of interferon-γ production was proposed to be mediated by a signaling pathway involving the p38 mitogen-activated protein kinase (Wang et al. 2004). FIP-gts was reported to significantly induce cytokine secretion, cellular proliferation in human peripheral mononuclear cells (HPBMCs) and interferon-γ expression. The effect of FIP-gts may be caused by the activation of phosphatidylinositol 3-kinase (Hsiao et al. 2008). Finally, the immunostimulus initiated by the recently isolated FIP-PCP is mediated via an enhanced production of NO, IL-1β, IL-6, IL-18 and TNF-α (Chang and Sheu 2007).

E. Industrial Production of Mushroom Immunomodulators

Immunomdulator metabolites can be isolated from fruiting bodies, cultured mycelia or culture filtrates. All medicinal mushrooms are lignocellulose degraders (white-rots, brown-rots, litter decomposers) and can utilize woody materials as growth substrates and for fruiting body production. One historical method of cultivation, that is still practiced mainly in Asia, is fungal cultivation on hardwood tree-logs. This process occurs over several years and yields two crops of mushrooms each year. It continues until the log physically "disappears" due to wood decay and lignocellulose decomposition. The use of polypropylene bags containing crushed lignocellulosics (including waste materials) and selected nutrients can be regarded as a modified version of the log method and actually represents a kind of solid-state fermentation (SSF). After autoclaving, the bags are inocultated with the mushroom mycelium of choice and can be incubated in the greenhouse under controlled conditions. This way, the production cycle for fruiting bodies can be shortened to 1–3 months (Smith et al. 2002). Mushroom production using SSF techniques was recently reviewed by Fan et al. (2008; see also Chapter 4 of this book). However, for the production of biomolecules, the production process should be carried out under more defined and controlled conditions to fulfil the strict requirements of the current good manufacturing practice (cGMP) for the production of active pharmaceutical ingredients (API). Quality control of mushroom cultivation poses several challenges, such as maintaining a constant substrate quality, temperature, moisture, a stable yield of the desired compounds and sterility.

To overcome these problems, more specific research was carried to cultivate mushrooms under submerged conditions. This method of cultivation has some advantages over SSF, for example, high yields in fungal mycelium under more defined conditions in a closed and well controlled

volume (higher space–time yields). Furthermore, sterility is easier to guarantee in a stirred-tank bioreactor than in logs or plastic bags (Lull et al. 2005). Nowadays, different bioactive metabolites from mushrooms can successfully be produced in submerged culture both in form of intracellular and extracellular products. However, in order to scale-up these methods to an industrial scale, various technical problems will have to be solved (Tang et al. 2007). Like in case of other fungal metabolites, medium composition governs the bioactive agents' production; for example, the production of mushroom polysaccharides was found to be regulated by the type and concentration of carbon and nitrogen souces (Wasser et al. 2003; Cui et al. 2006), the C/N ratio (Wang et al. 2005), by the ammonium ion concentration (Mao and Zhong 2006) and by different other components supplemented to the medium (Lim and Yun 2006). Other main key parameters influencing product yield are shear stress (Gong and Zhong 2005) and the mode of oxygen sypply to the culture (Tang and Zhong 2003). The steady-state concentration of the latter in the culture medium was found to be very important for the over-production of mushroom metabolites in bioreactors (Mao and Zhong 2004). Among different cultivation strategies applied, fed-batch cultivation was proved to be the method of choice for most mushrooms tested so far (Kim et al. 2006; Zou 2006). The recent reviews of Zhong and Tang (2004) and Tang et al. (2007) summarize and discuss the latest developments in this field.

IV. Conclusions

Fungal immunomodulators act either as suppressors or stimulators, and they are equally important molecules for many medical applications. The number of known fungal immunomodulators is increasing every year through the discovery of new compounds from so-far ignored fungal species and the development of appropriate semi-synthetic derivatives. This has led to a rapid expansion of the respective market. Some of these agents have approved from FDA and are widely used as commercial preparations, some have reached the level of clinical trials and others are currently subject of detailed pre-clinical tests. Also in the future, the search for new safe immunomodulators (without severe side-effects) to substitute the currently used preparations will be an important goal for both the scientific and the industrial communities related to the health care sector.

Mushrooms are one of the most promising sources for the production of safe and effective immunomdulators. The number of mushroom species ("macrofungi") on earth has been estimated to be 140 000, suggesting that only 10%

are known so far. Assuming that the proportion of "useful" mushrooms among the undiscovered and unexamined species will be only 5%, this implies a number of 7000 promising species as a future source of immunomodulators (Lindequist et al. 2005). Moreover, it has to been taken into consideration that different strains of one species can produce different bioactive compounds. For example, strains of *G. lucidum* can produce more than 120 different triterpenes and in addition also bioactive polysaccharides and proteins.

Based on the increased knowledge of the biochemistry and molecular biology of bioactive metabolites as well as the advanced technology of high-throughput screenings using omic approaches (genomics, proteomics, metabolomics), a rapid development of this research field is expected and will lead to the discovery of many novel immunomodulators in the near future. Not least, the investigation of novel immunomodulators and their effects will widen our knowledge of the complex mechanisms regulating the immune system and body defense.

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