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Antimycobacterials from natural sources: ancient times, antibiotic era and novel scaffolds

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1. ABSTRACT

Mycobacteria are a group of aerobic, non-motile, acid fast bacteria that have a characteristic cell wall composed of a mycolyl-arabinogalactan-peptidoglycan complex. They display different phenotypic attributes in their growth, color and biochemistry. Tuberculosis (TB) is defined as the infection with *Mycobacterium tuberculosis* complex and was declared a global health emergency principally because of the appearance of multidrug-resistant strains and the associated risk of infection in immune-compromised population. There is an urgent clinical need for novel, potent and safe anti-TB drugs. Natural products have been used since antiquity for treating diverse complaints and novel pharmacophores are discovered every year. Two of the most potent used antimycobacterials, the rifamycins and streptomycin, were first detected in *Streptomyces* bacteria. Plants are also the source of an exquisite variety of antimicrobials that can lead to useful therapeutics in the future. In this review, natural preparations used since antiquity for treating tuberculosis are described, together with a rapid view of the 20th century antibiotic development against TB. Finally a summary of the most potent recent natural antimycobacterials is displayed.

2. INTRODUCTION

It has been hypothesized that mycobacteria existed in the Jurassic geological period (1-2), however the imperfection of the fossil register and the difficulty of observing microorganisms in petrifications make the probable date of appearance of mycobacteria purely speculative. DNA from the *Mycobacterium tuberculosis* complex has been found in the bones of an extinct long-horned bison in Wyoming dating from 18,000 years ago (3). In Egyptian (4000 BC) and in Peruvian human mummies (1000 BC) mycobacterial DNA characteristic of the tuberculosis complex has also been found (4-5). Tuberculosis (TB) has been a dreadful disease since human history incipiency appearing in all geographical populations at all times. We should not forget the disastrous outbreak at the end of 19th century which may recall us today on the necessity of having a constant alertness towards the disease. Many famous writers, philosophers and poets (John Keats, Franz Kafka, Edgar Allan Poe, George Orwell, Karl Marx), artists (Paul Gauguin, Amadeo Modigliani), composers (Frederic Chopin, Carl PE Bach), and political leaders (Simon Bolivar, Cardinal Richelieu, King Tutankhamen of Egypt) succumbed to the disease (6). It was also the source of inspiration for literature, arts and

other human activities, and the pieces "The Magical Mountain" by Thomas Mann or the painting "Misery" by Cristobal Rojas clearly evidence this relation.

Today we encounter *Mycobacterium* species persisting in fish, amphibians, reptiles, birds, and mammals and their ubiquity in organisms as well as in the soil and the water, reflect their ancient past (7). It is possible that through their evolution, mycobacteria had to face nutrient depletion or poisonous environments and we can hypothesize that their complex cell wall is the consequence of such an overwhelming paleo-environment. Recently *Streptomyces* and *Mycobacterium* species were found to have a similar lifecycle (8) and perhaps *Streptomyces* developed the production of antibiotics as an alternative tactic to overcome highly competitive microbial environments. Mycobacteria is one of the most unsusceptible microorganisms against chemical injury, and the impermeability, thickness and low fluidity of its cell wall extensively contributes to its natural resistance against chemical agents (9).

The *M. tuberculosis* complex is a set of evolutionary closely related slow growing mycobacterial species, all containing the mobile insertion sequence IS6610 in their genome (10), and causing TB disease in humans and other mammals. TB is nowadays a growing global health problem since the numbers of human cases are increasing all around the planet. For 2009 the World Health Organization reported more than 9.4 million new cases and more than 1.6 million deaths (11). The main factors aggravating the issue are the massive appearance of drug resistant strains of *M. tuberculosis*: multi-drug resistant (MDR), resistant to isoniazid and rifampicin, and extensively-drug resistant (XDR), resistant to isoniazid, rifampicin, any fluoroquinolone and at least one second-line injectable drug, and HIV co-infection (12). Although there is an increasing funding trend for TB drug research (13) it is not clear whether there are enough novel chemical entities entering the pipeline to assure a new full chemotherapeutic regimen in the next decade (14-15). Novel drugs must comply with three conditions: safety, potency sufficient to shorten treatment and prevent rapid emergence of resistance, and absence of interaction with antiretroviral therapy. To achieve these goals several pharmaceutical companies (GlaxoSmithKline, Sanofi-Aventis, AstraZeneca, Bayer, Novartis) have entered in collaboration with academics from universities and small companies (FASgen Inc, Lupin Limited, Otsuka, Sequella Inc, Cumbre Pharmaceuticals) with the financial help of the TB Alliance (consortium sponsored by the Bill and Melinda Gates Foundation), for establishing the dynamic partnership necessary for urgently developing a new, effective and safe chemotherapy of TB.

3. ANCIENT TIMES

M. tuberculosis has infected *Homo sapiens* from the very beginning of human history (16). "Phthisis" (wasting) was the Greek name of the disease and "consumption" is a French-derived word (originally from the Latin word "consumere") which translates one of the features of the disease: the depletion of the body (17). In ancient Egypt, Greece and in the Roman Empire, the use of natural scents and essential oils was widespread, used often for baths and cleaning purposes and these practices can be considered as the beginning of aromatherapy. The Egyptians used cedar-wood oil and natural resins and ointments in a mixture known as "cedrium" for embalming and protecting the corpses from microorganisms and preventing the deleterious effects of oxygen and water (18). The Greek and the Roman civilizations inherited the medicinal legacy of the Egyptians and they increased our knowledge of medicinal plants enormously, notably with Pedanius Dioscorides´ (40-90 AD) famous book *De Materia Medica*. These ancient civilizations were also well aware of terrible diseases.

Hippocrates (460-376 BC) described phthisis as being almost always fatal, and recommended a special diet based on milk, wine and bread (19). Aristotle (384-322 BC) deducted the contagious nature of phthisis and Pliny (23-79 AD) attributed curative properties to pine forests (20). Propolis, the resinous material produced by bees from plant exudates, was used in ancient Greece for treating infections including tuberculosis (21), however its efficacy is still not conclusive (22).

Historically garlic (*Allium sativum*) has been one of the most useful natural medicines ever described and it has been employed by several ancient civilizations for different purposes (23). In ancient Greece, Rome, India and China it was used for treating infections and respiratory ailments, among other applications. More recently it has been proved that garlic inhibits the growth of almost all species of mycobacteria (24). Diallylthiosulfinate or allicin (1) (Figure 1, Table 1) is the phytochemical responsible for the antimicrobial action of garlic and it is generated by the enzyme alliin lyase from *S*-allylcysteine-*S*-oxide (alliin) when garlic cloves are crushed (25). However due to the instability of allicin further degradation products like ajoenes, vinyldithiin and sulfides (26) have to be taken into account when estimating the *in vitro* and *in vivo* activity of allicin. The minimum inhibitory concentration (MIC) of allicin has been reported to be 25 mg/L against both susceptible and isoniazid-resistant strains (27). RNA synthesis in *Salmonella typhimurium* was demonstrated to be inhibited by allicin (Figure 5) (28). Moreover allicin was shown to be toxic for mammalian phagocytic cells at a concentration higher than 100 μM, but at a lower concentration it decreases the expression of endogenous antigen for *M. tuberculosis* 85B together with TNF-γ, acting at the mRNA level (29-30). These results show that allicin has a dual action, acting directly on the microorganism by inhibiting its growth but also exerting an immunomodulatory action on the host. Furthermore other types of interesting antimycobacterials have been isolated from *Allium* species, such as the pyridine-*N*-oxides (31).

Chocolate, known also as "the food of the Gods", is obtained from the roasted seeds of the South American shrub *Theobroma cacao* (Sterculiaceae). The drink obtained from the slight roasting of the seeds is said to have healing properties that can alleviate those who are suffering

Table 1. Antimycobacterial data (MIC, SI) and targets of promising natural scaffolds and antibiotics

Natural product class	Compound	Antimycobacterial data		Molecular antimycobacterial target	References
		MIC on *M. tuberculosis* or specified (mg/L)	Selectivity index in relation to mammalian cells (SI = GIC_{50} / MIC)		
	Allicin (1)	25	0.65	RNA synthesis inhibition	(27, 29)
	Licochalcone A (2)	20	nd	Unknown	(41)
	Glabridin (3)	30	nd	Unknown	(42)
	Caespitate (4)	100	nd	Unknown	(46)
	(*E*)-phytol (5)	2	nd	Unknown	(48)
	Lawsone (6)	100	nd	Unkownn	(51)
	(*3R,8S,9Z*)-Falcarindiol (7)	8 Mb	0.6	Unknown	(54)
	Berberine (8)	25 Ms	nd	Exact mechanism unknown	(59, 61)
Antibiotics	Benzylpenicillin (9)	> 512 Mf	nd	Peptidoglycan biosynthesis inhibition	(77)
	Streptomycin (10)	1	nd	16S rRNA of 30S ribosomal subunit	(85)
	Kanamycin (11)	2	nd	16S rRNA of 30S ribosomal subunit	(93)
	Amikacin (12)	0.75	nd	16S rRNA of 30S ribosomal subunit	(97)
	Cycloserine (13)	25	nd	Alanine racemase (Alr) and L-ala:D-ala ligase (Ddl)	(105)
	Rifampicin (14)	0.25	nd	B-subunit of RNA polymerase (RpoB)	(114)
	Capreomycin (15)	2	nd	70S ribosomal subunit	(118)
	Clarithromycin (16)	8	nd	50S ribosomal subunit	(128)
Lipids, sterols and triterpenoids	(*R/S*)-2-methoxydecanoic acid (17)	44	1.5	Unknown	(161)
	2-octadecynoic acid (18)	2.5 Mb	nd	Unknown	(163)
	Micromolide (19)	1.5	63	Unknown	(166)
	24,25-Epoxycyclo-artan-3-one (20)	8	9	Unknown	(168)
	3-Epi-lupeol (21)	4	15	Unknown	(172)
	Saringosterol-24-epimers (22)	0.125	1024	Unknown	(173)
	Stigmast-5,22-dien-3β-ol-7-one (23)	4	95	Unknown	(174)
Terpenoids	Dehydrocostus lactone (24)	2	nd	Unknown	(183)
	12-Demethyl-multicauline (25)	0.46	nd	Unknown	(186)
Phenolics	7-Methyljuglone (26)	0.5	30	Micothiol disulfide reductase (Mtr)	(189-192)
	Engelhardione (27)	0.2	nd	Unknown	(199)
	Bakuchiol (28)	20 Mb	nd	Unknown	(202)
	Preussomerin (29)	3	6	Unkown	(206)
Alkaloids	Ascididemin (30)	0.1	0.4	Mycobactin biosynthesis (MtbB)	(209-210)
	Sampangine (31)	0.2 Mi	24	Unknown	(212)
	Evocarpine (32)	2 Ms	nd	Unknown	(214)
	Hirsutellone A (33)	0.78	64	Unknown	(216)
	6-Hydroxy-manzamine E (34)	0.4	11	Unknown	(217)

Mb *M. bovis* BCG; Mi *M. intracellulare*; Ms *M. smegmatis*; Mf *M. fortuitum*. nd *no data*

from tuberculosis (32). There is no information about the antimycobacterial activity of the pyrones, pyrazines, furans and other products formed during the roasting of the cocoa beans; however a report in 2008 showed that roasted cocoa beans have radical scavenging (antioxidant) and antibacterial activities (33).

In Ayurvedic medicine, two plants have been used since antiquity for treating tuberculosis: *Tylophora asthmatica* (Asclepiadaceae) and *Ocimum sanctum* (Lamiaceae) (34). Leaves of *Tylophora asthmatica* also recorded as *Tylophora indica*, are a major source of phenanthroindolizine alkaloids (35) however reports of the anti-tubercular activity of this type of alkaloid have not been found, probably because they have not been tested or because they are inactive. *Ocimum sanctum* is known by the local communities as "Tulsi" or holy basil and the extract of this plant has shown inhibition of *M. tuberculosis* growth, but again no active compound has yet been isolated (36). Another plant widely used in traditional Ayurvedic medicine is *Adathoda vasica* (Acanthaceae) known as "Vasaka" for curing bronchitis, leprosy, tumors, fever, gonorrhea and tuberculosis among other ailments (37). The water extract of the leaves of this plant was shown to have antimycobacterial activity but a detailed phytochemical evaluation is lacking (38). "Mandukaparni" or "Gotu Kola" are names given to the Ayurvedic rejuvenator plant *Centella asiatica* (Apiaceae) which has been recommended for tuberculosis, leprosy, diarrhea, inflammation, and psoriasis among several ailments (39). Asiaticoside and other triterpene glycosides have been isolated from the roots of *Centella asiatica*, and there is one report of its inhibitory activity against *M. leprae* and *M. tuberculosis* (40), showing an MIC value greater than 16 mg/L with no mention of cytotoxicity. Licorice or *Glycyrrhiza glabra* (Fabaceae) is another old traditional herb described in Ayurveda, both as a medicinal and as a flavoring resource. Licochalcone A (2) was isolated from Chinese licorice roots and was initially thought to be responsible for the antimycobacterial activity of the plant, having an MIC less than 20 mg/L against species of the *M. tuberculosis* complex (41). However, later it was found that another phytochemical with an interesting antimycobacterial profile could also contribute to the antimycobacterial properties of

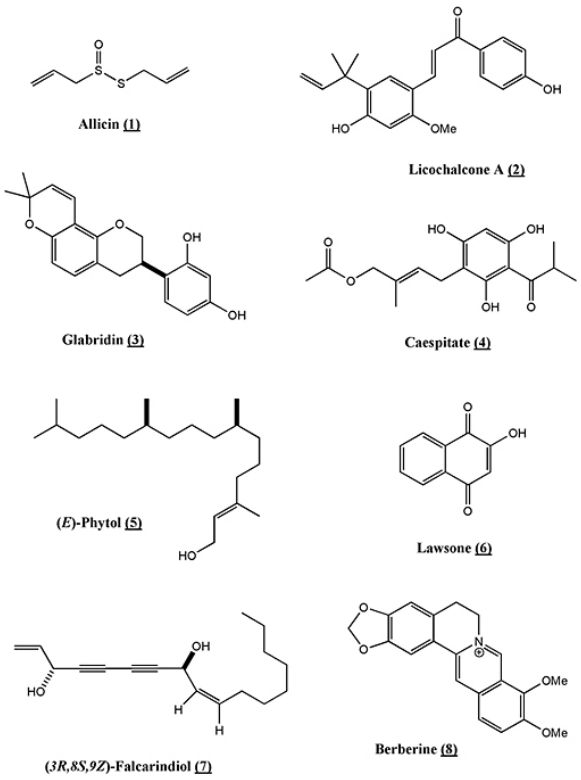

Figure 1. Chemical structure of some active antimycobacterial natural products obtained from ancient medicinal plants.

this plant. This compound, glabridin (3), was isolated from the ethyl acetate extract of the roots of *Glycyrrhiza glabra* collected in India and it showed an MIC of 30 mg/L against *M. tuberculosis* $H_{37}Rv$ (42).

Traditionally in Africa, species of the genus *Helichrysum* (Asteraceae) have been used for the treatment of respiratory disorders and tuberculosis (43-44). Whilst some compounds have been isolated from these species, none have shown potent inhibition of mycobacterial growth (45). Until now, the most active compound found in *Helichrysum caespititium* is caespitate (4) which has an MIC value of 100 mg/L against *M. tuberculosis* (46). In a review of the plants used in South African traditional medicine for treating mycobacterial infections, several species of the *Helichrysum* genus are reported to be used for treating coughs and TB (47). Interestingly the Asteraceae family is recorded to have prominent application for coughs, chest and respiratory complaints related to TB symptomatology and therefore the Asteraceae species should be considered for bio-guided phytochemical studies. The infusion of the leaves of the Kenyan shrub *Leucas volkenssii* (Lamiaceae) has been used as a remedy for asthma, bronchitis and other lung diseases. A bioguided phytochemical study showed the presence of several lipophilic components with very potent inhibition of mycobacterial growth. In particular (*E*)-phytol (5) showed an MIC value of 2 mg/L against *M. tuberculosis* $H_{37}Rv$ in a radiorespirometric assay (48). The leaves of *Lawsonia inermis* (Lythraceae) produce a dye known as "Henna" which has been widely used for body art since antiquity and was reported as a treatment for leprosy (49). An infusion of the leaves is able to reduce the number of lesions by 30% in relation to the control, notably in the kidneys and liver, in guinea pigs infected with *M. tuberculosis* (50). The phytochemicals assumed to be responsible for the activity are of quinoid nature such as lawsone (6) which has demonstrated an MIC value close to 100 mg/L against *M. tuberculosis* (51). The antimycobacterial activity of naphthoquinones has motivated a deeper research focusing on the determination of the chemical features essential for their activity, leading to active derivatives which will be discussed later.

In China, during the 28th Century BC, Shen Nong wrote a book titled "Pen-T´sao" (translated as "The Herbal") describing hundreds of herbal medicines and poisons mostly tested directly on himself (52). The herb known as "Bai-Zhi" corresponds to *Angelica dahurica* (Apiaceae) used for the relief of headaches, colds and infections (53). An active polyacetylene known as (3*R*,8*S*)-falcarindiol (7) has been isolated from the roots of this plant, displaying an MIC value as low as 8 mg/L against *M. fortuitum* (54) and *M. bovis* BCG. Moreover falcarindiol has also a marked mammalian cytotoxicity of the same order of magnitude as the antibacterial activity (55), therefore preventing its use as antimicrobial. The molecular target of falcarindiol is still unknown although several studies point to an induction of anti-oxidant enzymes at low concentrations which are beneficial but with a reversion of the effect at higher concentrations (56-57). The rhizome of *Coptis chinensis* (Ranunculaceae) know as "Huang Lian" which means "yellow connection" is a very bitter root that has been extensively used for treating diarrhea, nausea, insomnia, and cleaning abscesses, furuncles, burns and other lesions (58). The active principle found in the "Huang Lian" rhizome is the quaternary alkaloid berberine (8), which has an MIC of 25 mg/L against fast-growing *M. smegmatis* but has a higher MIC against pathogenic mycobacteria such as *M. avium* (MIC 50 mg/L) and *M. bovis* BCG (MIC 200 mg/L) (59). Berberine is a compound with multiple biological activities, acting as immunomodulatory being able to activate macrophages (60), it also intercalates in DNA (61) and it prevents the adherence of microorganisms to the cells (62).

During the obscure period of the Middle Ages (5th to 15th Century) very little advance of biosciences was achieved. The clergy kept the knowledge of the herbs in the convents and also the majority of medieval people were analphabet. By the turn of the 16th Century and the introduction of liberal Renaissance ideas notably in the arts, coupled to the discovery of a new continent, logical and scientific thinking started to appear again. South American *Cinchona* bark and Ipecacuanha root were transported to Europe for treating malaria and dysentery respectively. Dysentery was a common problem in the days of unsanitary urban settings in which people would throw dirty water and rubbish into the streets. Some scientific spirits tried to demystify the real world by inventing novel instruments and postulating novel theories to explain it.

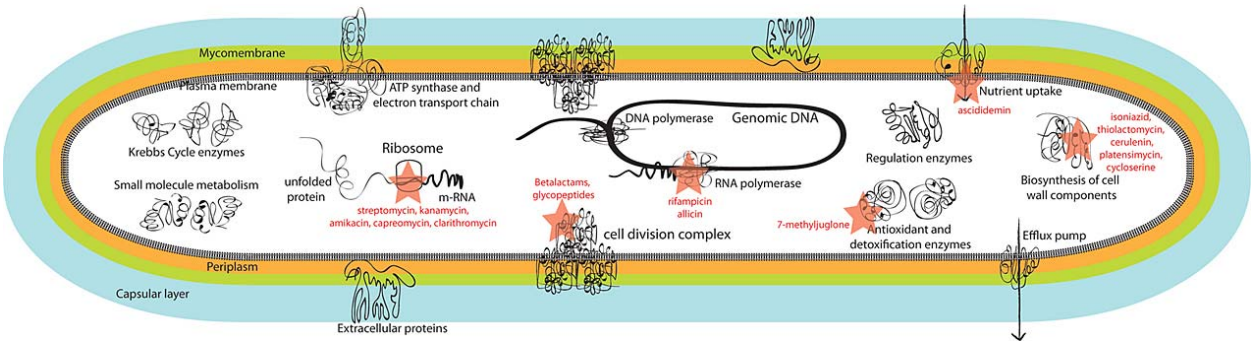

Figure 5. Mycobacterial cell diagram showing the principal biochemical pathways and natural product inhibitors.

Notably the experimental sciences: physics with Galileo Galilei (1564-1672), Johannes Kepler (1571-1630) and Isaac Newton (1643-1727) and chemistry with Robert Boyle (1627-1691), Antoine Lavoisier (1743-1794) and Jacob Berzelius (1779-1848) started to shape the thinking of academics in Europe, providing novel ideas for structuring a modern view of the world.

4. DISCOVERY OF ANTIBIOTICS

Louis Pasteur (1822-1895) was a French chemist, microbiologist, immunologist and biotechnologist who brilliantly tackled many important problems of the biosciences of the epoch. Amongst his most important achievements are the foundation of chemical stereochemistry, the introduction of the germ theory of diseases, the demonstration of microbial fermentation and the setting of the first vaccines against virus and bacteria (63). Together with Antony van Leeuwenhoek (1632-1723), the first to observe under the microscope "animalcules" or microorganisms, Louis Pasteur is considered the father of microbiology. Later Joseph Lister (1827-1912), an English surgeon, was convinced by the experiments of his French contemporary, of the necessity of an aseptic technique in surgery and he was the first physician to use antiseptic (64). Lister used carbolic acid, which is known today as phenol, and he described it, in 1867, as a "volatile organic compound which appears to exercise a peculiarly destructive influence upon low forms of life" (65). The word "antibiosis" was used for the first time in 1889 by Pasteur´s pupil Paul Vuillemin (1861-1932) for describing the action of one organism against another one in mixed cultures (66). The pioneer work of chemotherapy can be ascribed to Paul Ehrlich (1854-1915) who was the first to notice that certain dyes colored selectively certain animal and bacterial cells while others did not. He realized that it may be possible to produce chemical compounds (dye like) known as "magic bullets" that selectively kill the bacterium while not killing the humans infected with the bacterium (67). With the help of Sahachiro Hata (1873-1938), the team chemically modified several dyes and salvarsan-606, an organo-arsenical compound, was successfully identified as a compound able to kill *Treponema pallidum*, the spirochaete bacterium that cause syphilis, without harming the host (68). This was the start of the chemotherapeutic era.

Undoubtedly a major breakthrough in the history of Medicine was the demonstration of the bacterial etiology of TB in Berlin in 1882 by the German physician Robert Koch (1843-1910) (69). He proposed "Koch´s Postulates" which are in fact a rational experimental design for proving that a bacterium is the cause of a disease (70). The first postulate establishes that in every case of the disease, the infecting organisms must be present. It must be possible also to isolate the microorganism causing the disease from a host carrying the disease and to grow it in pure culture. Then it must be shown that after inoculation of the pure culture on a healthy susceptible host, the disease appears. Finally the organism causing the disease must be re-isolated from the experimentally infected host. Although the French military physician Jean-Antoine Villemin (1827-1892) had already demonstrated that TB was contagious using rabbits (71), it was Robert Koch who isolated, cultured and stained *M. tuberculosis*. He successfully inoculated guinea pigs and re-isolated the mycobacterial strain from the organs of the sick animals. Finally Koch was the first to prepare tuberculin, an extracellular protein mixture extracted from cultures of *M. tuberculosis*, which he claimed erroneously to be the cure of TB as a vaccine (72), but which was shown to be a very useful diagnostic test developed years later by Clemens von Pirquet (1874-1929) and established finally by Charles Mantoux (1877-1947).

Although Ehrlich´s salvarsan was effective in curing spirochaetal infection it was also considerably toxic and undesirable side effects were common (73). However it was used globally as the only cure for syphilis until the advent of the first antibiotic. In 1928, the Scottish microbiologist Alexander Fleming (1881-1955) discovered a lysogenic effect on an abandoned Petri dish of staphylococci contaminated by the fungi *Penicillium notatum*, when returning from a holiday. By analytical observation, he hypothesized that the fungi produced a chemical which diffused slowly on the plate and attempted to isolate the potent compound. The substance was soluble in ethanol but not in water, discarding the possibility of a lysogenic protein such as lysozyme (74). Ernst Chain (1906-1979) and Howard Florey (1898-1968) were able to isolate around 1940, a mildly pure brown penicillin sample from a fermented culture of the fungi. It was however Gerhard Domagk (1895-1964) and his team who experimentally obtained the first agent active against

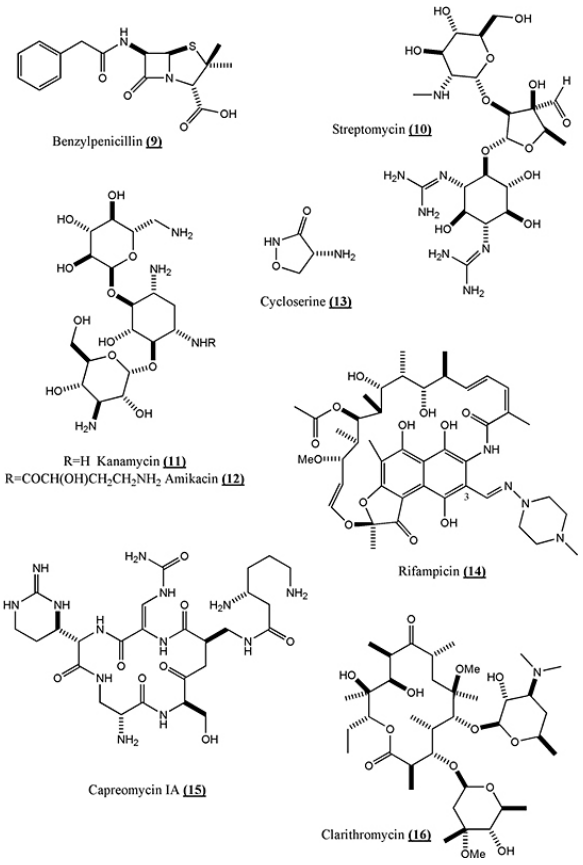

Figure 2. Antibiotics used in the treatment of mycobacterial diseases (except benzylpenicillin) developed from natural products discovered in the antibiotic era.

streptococci (the microbes causing bacterial sore throat) and staphylococci. Synthesized in 1932 by I.G. Farbenindustrie, the so called "Prontosil Rubrum", was the first sulfonamide ever used on patients, and it saved 6-year old Domagk´s daughter, Hildegard, from arm amputation following a streptococcal infection (75). From natural sources, other antimicrobials were soon discovered such as gramicidin and tyrocidine isolated by the French microbiologist René Dubos (1901-1982) from cultures of the soil inhabitant *Bacillus brevis* (76). However these compounds were either inactive against *M. tuberculosis* or the concentration needed to kill the bug was so high that toxicity hindered their use.

The penicillins, cephalosporins, carbapenems and monobactams are arguably the most important group of antibiotics, characterized by the presence of the fused β-lactam which prevent the final crosslinking and polymerization steps of the biosynthesis of peptidoglycan (Figure 5). Benzylpenicillin or penicillin G (9) (Figure 2, Table 1) was the first naturally occurring β-lactam to be discovered, however it has a weak activity against mycobacteria (>512 mg/L for *M. fortuitum*) (77) due to the fact that mycobacteria produce chromosomally encoded β-lactamases (78). It has been suggested therefore that a mixture of a β-lactamase inhibitor (clavulanic acid) together with a β-lactam might be useful for treatment of TB and other mycobacterial diseases (79-81) however conclusive clinical studies are still expected. Clavulanic acid has been hypothesized to have also a direct effect on the cell wall facilitating the entry of β-lactams to the periplasmic space (82).

The first natural product to be successfully used for curing TB patients was streptomycin (10), an aminoglycoside isolated from *Streptomyces griseus* by Selman Waksman (1888-1973) and Albert Schatz (1922-2005) at Rutgers University in 1943 (83). The word "antibiotic" was clarified by Waksman to delimit the group of "chemical substances, produced by microorganisms, which have the capacity to inhibit the growth of and even to destroy bacteria and other microorganisms" (66). Waksman places emphasis on the selective character of the antibiotics, some of them been able only to kill a specific type of microorganisms while others have a wider spectrum of activity. In March 1944, streptomycin was tested on animals by Merck & Co in the Mayo Clinic and in 1946 the UK Medical Research Council performed a randomized trial of streptomycin on young individuals with acute, bacteriologically proven pulmonary TB, achieving cure rates of 100% (84). The impact of streptomycin was seen as miraculous particularly on children with TB meningitis; however the joy was soon eclipsed as many children relapsed after a few months of treatment. It was evident that *M. tuberculosis* had become resistant to streptomycin. The MIC of streptomycin for susceptible *M. tuberculosis* strains is between 1-2 mg/L and between 25-50 mg/L for resistant strains; with 30 mg/L being the critical concentration, defined as the concentration able to inhibit 95% of the wild-type strains while not inhibiting the resistant strains (85). A study by the British bacteriologist Dennis Mitchison (born in 1919) showed that streptomycin-resistant variants appeared in populations of *M. tuberculosis* that had never been exposed to the drug with a discontinuous distribution (86). At low populations (less than 10^6 cells), all the bacteria were susceptible to 2 mg/L of streptomycin, however when the population reached 10^9 cells, several hundreds of cells were able to grow at 10 mg/L of streptomycin and around 1-2 cells at a concentration of 100 or 1000 mg/L of streptomycin. These bacterial populations are commonly found in TB lesions and therefore in monotherapy, resistance develops quite rapidly. It is recognized today that the molecular target of streptomycin is the small ribosomal unit known as S12 codified by the *rpsL* gene, which contains several mutations in streptomycin resistant strains of *M. tuberculosis* (87).

In 1940, Frederick Bernheim discovered that the TB bacillus doubled the uptake of oxygen when sodium salicylate was present in the media (88). We have to remind the reader that salicin is the glycoside of salicylic alcohol, occurring naturally in many *Salix* species (Salicaceae). Bernheim published a second paper disclosing that 2,3,5-triiodobenzoate and nicotinic acid inhibited the growth of *M. tuberculosis* (89). It was the Swedish chemist Jörgen Lehmann (1898-1989) who, after reading the 1940 paper of Bernheim, realized that this meant that salicylic-type

structures had some specific effect "on the internal workings of the tuberculosis bacterium – something that might be significant for its very life" (67). Allied with the small Swedish pharmaceutical company Ferrosan, he predicted that the presence of an amino group in the *para* position of the carboxylic acid of salicylic acid would have an inhibitory effect of TB bacillus. By December 1943, with minute amounts of the drug, Lehman confirmed that he was indeed correct (67). The MIC of *p*-aminosalicylic acid is 0.3-1 mg/L against *M. tuberculosis* $H_{37}Rv$, and it has proven to be a very effective second-line anti-TB agent despite its irritant and nauseous side effects (90).

After the Second World War, Japan organized the "Japan Antibiotics Research Association" in order to recruit experts in microbiology, fermentation and natural product chemistry for the manufacture of penicillin (91). In the golden era of antibiotics (1950-1965), several Japanese groups started exploratory research in microbial products disclosing a vast array of antibiotic classes such as colistin (1950), variotin (1951), trichomycin (1952) gramicidin J (1952), kitasamycin (1953) and the mikamycins (1956). In 1957 Hamao Umezawa (1914-1986) isolated kanamycin (11) from *Streptomyces kanamyceticus* (92) and was soon established as the drug of choice for treatment of streptomycin-resistant TB. The MIC of kanamycin against *M. tuberculosis* $H_{37}Rv$ is close to 2 mg/L and it inhibits protein synthesis by tightly binding to 16S rRNA of the 30S ribosomal unit (Figure 5) (93). Kanamycin-resistant strains have an MIC value of more than 200 mg/L and this resistance can be caused by three different mechanisms: enzymatic aminoglycoside modification, specific methylation of the rRNA or mutation in the 16S rRNA codifying gene *rrs* (94). The clinical adverse reactions of aminoglycosides are typically nephrotoxicity and ototoxicity caused by a perturbation of the membrane function of the cells, leading to damage of the renal tubule and the vestibule followed by hearing loss (95). Amikacin (12) a semisynthetic derivative of kanamycin was synthesized in 1972, in order to generate an analog of kanamycin with activity towards kanamycin-resistant strains (96). *N*-acetylation, *O*-phosphorylation and *O*-adenylation are mechanisms for the enzymatic modification of aminoglycosides rendering them inactive, and amikacin was developed in order to prevent *N*-acylation. Amikacin has an MIC between 0.5-1 mg/L against *M. tuberculosis* $H_{37}Rv$ and has been shown to be superior to kanamycin with a bactericidal activity at 2 mg/L (97). It has also been demonstrated that all amikacin-resistant strains are resistant to kanamycin, but not all kanamycin-resistant strains are resistant to amikacin supposing a more difficult step for the bacteria to become resistant to amikacin (98). A typical mutation conferring resistance to both kanamycin and amikacin occurs in the *rrs* gene, replacing adenine in position 1401 for guanine.

It is not clear whether the German group supervised by Gerhard Domagk or the North American team at Squibb and Hoffman-La-Roche were the first to test the activity of the isomers of nicotinic acid (99). Based on early observations that nicotinamide (vitamin B3) had anti-tubercular properties, researchers worldwide were exploring this particular class of nitrogen heterocycle simultaneously (100). Both pyrazinamide and isoniazid were developments of the same chemical lead. Although Roche and Squibb held the patent for isoniazid, they did not retain the exclusive rights and granted production licenses to reliable pharmaceutical companies in order to increase the coverage and the supply of the drug all over the world (101). Today it is recognized that isoniazid is the most popular anti-tubercular agent, for *M. tuberculosis* is extremely sensitive to this synthetic chemical, admittedly as a consequence of a pleiotropic effect (102-103). It has been demonstrated that flavonols can significantly reduce isoniazid resistance in rapid growing mycobacteria (104).

Almost at the same time, another interesting antitubercular compound made its appearance. Cycloserine (13) was isolated in 1952 from *Streptomyces orchidaceus* by Commercial Solvents Corp. This isoxazolidinone has wide spectrum of activity against Gram-positive and Gram-negative bacteria and particularly against mycobacteria. Cycloserine inhibits two enzymes alanine racemase (Alr) and D-alanine D-alanine ligase (Ddl) of the peptidoglycan biosynthesis pathway (105). The MIC of cycloserine is 25 mg/L against the $H_{37}Rv$ strain. Despite being an efficient agent, its severe toxicity has limited its widespread use and it is included as a second line drug (106). There are also other antibiotics acting on the cytoplasmic steps of the biosynthesis of peptidoglycan. Fosfomycin is an inhibitor of the MurA ligase of *Escherichia coli*, forming an adduct with a cysteine residue of the protein (107). In the MurA of *M. tuberculosis*, the critical residue is an aspartic acid and it is assumed that *Mycobacterium* has innate resistance to fosfomycin. Another interesting antibiotic is bacitracin which has a more pronounced inhibitory effect on *M. smegmatis* than *M. tuberculosis* (108). It functions by sequestering undecaprenol phosphate by forming a complex together with a bound divalent metal cation, therefore preventing its binding with a phosphatase and finally inhibiting peptidoglycan biosynthesis (109).

Streptomyces mediterranei was initially obtained from a pine forest soil sample near Nice in 1959 in France by Lepetit Research Laboratories (110). The name of the derived antibiotic rifamycin was coined from a French popular movie of the time "Rififi", perhaps translating the difficult and complex isolation and characterization of the active products. The isolated rifamycins A, B, C, D and E were separated according their mobility in paper chromatography (111). The naturally occurring rifamycin B can be oxidized reversibly to rifamycin O which then loses a glycolic acid molecule liberating rifamycin S, which is very active (112). However these naturally occurring rifamycins have poor solubility in water and low absorption, and therefore cannot be administered orally. Several hundred rifamycin analogues were prepared by Lepetit and Ciba and it was found that the introduction of a formyl group (or another unsaturated substituent) in position 3 usually increased the antibacterial activity of the initial rifamycin. In 1966 rifampicin (14) was finally formulated as an oral, safe and effective anti-TB drug (111). Rifampicin acts on the β-unit of RNA polymerase (Figure 5) codified by *rpoB*, preventing DNA translation to

RNA. Crystals of the *Thermus aquaticus* RNA polymerase bound to rifampicin were obtained by the soaking technique and showed that rifampicin binds to a deep pocket within the DNA/RNA channel around 12 Å from the active site, therefore preventing elongation of the RNA polymer (113). The MIC of rifampicin is 0.1-0.4 mg/L for *M. tuberculosis* $H_{37}Rv$ (114). Almost all of the mutations conferring rifampicin resistance occur on rpoB (> 98%) notably in the region from 513 to 531 amino acids which provide high levels of resistance. There are other analogues of rifampicin such as rifabutin and rifapentine, but resistant *M. tuberculosis* strains can show cross-resistance, rendering these compounds ineffective (115).

Capreomycin (15) is a cyclic pentapeptide antibiotic isolated by Herr and collaborators from *Streptomyces capreolus* in 1960 (116). It is a water soluble compound with close chemical and biological properties to viomycin and the tuberactomycin antibiotics (117). Commercially available capreomycin occurs as a mixture of closely related IA and IB differing by the presence of serine in capreomycin IA instead of alanine in IB. The MIC of capreomycin IA is 2 mg/L against *M. tuberculosis* $H_{37}Rv$ and it is also active against *M. avium* complex species (118). The importance of capreomycin resides on its ability to inhibit the growth of multi-drug resistant strains and non-replicative forms (119). Although the exact mechanism of action is still unknown, it is clear that it interacts with the ribosome by inhibiting translation and therefore protein synthesis. By using microarrays, the translational response of *M. tuberculosis* was studied, and the results showed that several genes are up-regulated following capreomycin exposure, notably the genes related to information pathways involved in the translational system, most encoding ribosomal proteins and t-RNA or r-RNA (120). The *tlyA* gene, although not essential, is thought to codify for a protein able to alter ribosomal proteins and plays a role in *M. tuberculosis* resistance to capreomycin (121). This antibiotic has the same toxicity profile as the aminoglycosides causing ototoxicity and nephrotoxicity, but can also cause renal tubulopathy characterized by alkalosis (122). Another interesting glycopeptide is vancomycin, which shows an MIC of 40 mg/L against the $H_{37}Rv$ strain and is thought to act by a pleiotropic mechanism, because several biochemical pathways are induced after exposure to inhibitory and sub-MIC levels of the antibiotic (123).

Pikromycin was the first macrolide antibiotic extracted from a natural source. It was isolated from *Streptomyces venezuelae*, taken from a Venezuelan soil sample in 1947. This species of *Streptomyces* also produces the important antibiotic chloramphenicol (124). The term "macrolide" was introduced in 1957 by Robert B. Woodward (1917-1979) as a shortcut of macrolactone glycoside to designate a class of polyketide antibiotics composed by a macrolactone ring bound with one or several aminosugar units (125). Pikromycin is a 14-membered macrolide reported for the first time in 1950 having weak activity against Gram-positive bacteria (126). Two years later, McGuire and collaborators isolated erythromycin A from *Saccharopolyspora erythraea* with an increased activity against Gram-positive and Gram-negative bacteria including *Legionella pneumophila*, *Mycoplasma pneumoniae* and *Chlamydia* spp (127). The second generation of macrolides was developed for increasing the bioavailability and pharmacokinetic profile of erythromycin (125). Clarithromycin (16) was prepared by a short sequence of chemical transformations from erythromycin, providing modifications that confer greater resistance towards acid-catalyzed inactivation. It has also increased lipophilicity which enhances tissue penetration and has been therefore effective against intracellular pathogens such as *Heamophilus influenzae* and *M. tuberculosis*. Clarithromycin shows an MIC value of 8 mg/L against the $H_{37}Rv$ strain (128), however there are reports of much weaker activity of clarithromycin against clinical isolates, with MIC values above 64 mg/L (129). In general clarithromycin is much more active against *M. avium* (MIC 8 mg/L), *M. kansasii* (MIC 0.5 mg/L) and *M. ulcerans* (MIC 2 mg/L) (130). Presently there is an increasing interest in developing further the macrolide class for increasing its potency specifically against *M. tuberculosis* (131-132).

There are several antibiotics that display inhibition on lipid biosynthesis of *M. tuberculosis* and this pathway may be important for future TB drug development. Thiolactomycin, isolated in 1982 from a *Nocardia* species (133), has shown an MIC of approximately 26 mg/L for the $H_{37}Rv$ strain (134). The mechanism of action has been reported to be by inhibition of β-ketoacyl-ACP synthases, KasA and KasB, which are necessary enzymes for the biosynthesis of mycolic acids. Cerulenin was initially obtained from *Cephalosporium caerulens* in 1963 and this compound demonstrated antimycobacterial activity, having an MIC value between 3.0-6.25 mg/L against *M. tuberculosis*, 1.5 mg/L against *M. smegmatis*, and 12.5 mg/L against *M. kansasii* (135). Different lipidic patterns were observed in treated cells of *M. bovis* BCG, *M. avium* and *M. smegmatis* in comparison with the controls, revealing that cerulenin acts on lipid biosynthesis. Platensimycin is another interesting antibiotic acting on lipid biosynthesis discovered in 2006 from *Streptomyces platensis* (136). By using an antisense method for comparing the level of RNA expression of fatty acid biosynthesis genes against controls after exposure to a wide range of natural extracts, Merck Co. found that an extract from a soil sample from Africa contained amounts of an active amide named platensimycin (137). The MIC of this compound against *M. tuberculosis* $H_{37}Rv$ is around 12 mg/L and it acts by inhibiting β-ketoacyl-ACP synthases just as thiolactomycin (138).

5. NOVEL ANTIMYCOBACTERIAL SCAFFOLDS FROM NATURAL SOURCES

In this part of the review we focus our attention on the natural products that are potent enough (MIC < 5 mg/L) to be considered as hits for generating novel antimycobacterial leads, displaying also an acceptable selectivity towards *M. tuberculosis* $H_{37}Rv$ (SI >10). Several bioguided studies have appeared in the literature showing inhibitory data for the isolated natural products, but only a

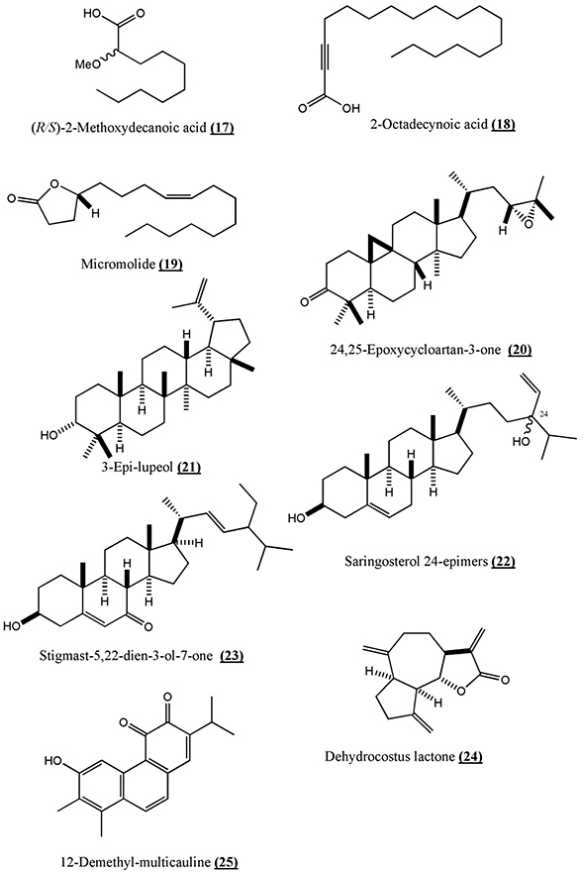

Figure 3. Fatty acids, lipids and terpenoidal natural products with antimycobacterial activity.

small fraction contains information about the non-specific toxicity towards mammalian cells. We insist on the fact that the MIC values only show the potency of the compound against bacterial viability but do not demonstrate selectivity. There are many chemicals which are highly toxic for both prokaryotic and eukaryotic cells and conversely there are other compounds which are mildly active as antibacterials yet having very low or no toxicity towards mammalian cells. These compounds would be easily ignored in a screening where only the MIC value is considered for compound selection and further development. Thus the determination of the half growth inhibitory concentration (GIC_{50}) against mammalian cells on early stages of drug discovery is likely to increase the impact of the first-time reported hits. An excellent review from 2005, emphasizes the importance of determining the cytotoxicity as early as possible (139). Many papers include the MIC of potent antimycobacterial compounds but lack the cytoxicity information, making the examination and comparison of hits a rather flawed and non-standardized procedure. A commentary from 2001 established that a promising hit should show a selectivity index (SI), defined as the ratio between the GIC_{50} on mammalian cells and the MIC on pathogenic mycobacteria, of more than 10 (140). That means that a preliminary hit must be selectively active against mycobacteria at least one order of magnitude in comparison with the degree they affect mammalian cells. Isoniazid is until now an "unbeatable" control, as it has a selectivity index superior to 40,000 (141). The 2001 commentary also encourages rapid determination of the viability of *Mycobacterium* inside the macrophages treated by inhibitors, in order to have a better picture of the intracellular action of the compound, the effective killing concentration inside the cell, and the transport mechanisms from the exterior to the interior of the mammalian cells (140).

There are few reviews on the antimycobacterial activity of natural products, some focusing on certain type or origin of metabolites such as terpenoids (142), marine natural products (143) and alkaloids (144) while others specialize on the coverage of plant metabolites (145) and others include many classes of natural products (146-148).

Mycobacteria has a complex and well-organized system for lipid acquisition and trafficking, being able to capture lipids from the host and utilize them as carbon source and energy (149-151). Around one tenth of all the functional genes of the genome of *M. tuberculosis* are related to lipids (152). Many mycobacterial proteins acting on lipid substrates have been predicted from the genome, involved in fatty acids degradation (118 open reading frames (ORFs)), lipid biosynthesis (65 ORFs), esterases and lipases (38 ORFs) and fatty acid transport (2 ORFs). Therefore it is not surprising that several lipid-like molecules have been found to have an inhibitory effect on the growth of *M. tuberculosis*.

There are early reports of the inhibitory action of oleic acid on TB bacilli from 1926 to 1950 by Boissevain, Platonov, Bergstrom, Dubos and others (153). In 1948, Sattler and Youmans noted that the wetting agent Tween 80 was able to inhibit the growth of *M. tuberculosis* specially when the inoculum size was small (154). The effect can be reversed if oleic acid is eliminated from impure Tween 80 or if albumin is added to the medium. As Tween 80 can be hydrolyzed by the bacteria to produce minute amounts of oleic acid, it can have inhibitory effects in the absence of albumin. By 1977 it was found that a free carboxylic acid was required for antimycobacterial activity, being their esters inactive (155). The most bactericidal fatty acid was found to be myristic acid (C14:0). Against rapid growing mycobacteria such as *M. aurum*, *M. parafortuitum*, *M flavescens* and *M. gilvum*, lauric acid (C12:0) was found to have an MIC value close to 6.25 mg/L (156). It has been demonstrated that activated macrophages release around six times more fatty acids than their normal non-activated macrophages, suggesting that macrophages can perform antimycobacterial functions without phagocytosis (157). Against mycobacteria belonging to the *M. avium* complex, the fatty acids lauric (C12:0), oleic (C18:1) and linolenic (C18:3) have comparable potency (158). When using a bioguided isolation method, one has to be aware that fatty acids frequently occur in lipophilic extracts and therefore dereplication procedures of active lipophilic extracts is recommended for avoiding isolating already known active components.

Interestingly ozonized sunflower oil (Oleozon) was found to have antibacterial properties with an MIC of

950 mg/L against *M. tuberculosis* $H_{37}Rv$ and other mycobacteria (159). The concentration may seem high in comparison with potent antibiotics but it must be remembered that the mixture is simple culinary oil and it has therefore little toxicity. The presumed mechanism of action of these fatty acids is by insertion into the mycolic or phospholipid layers of the bacterial cell walls, therefore altering their permeability and function (156).

The structural type of 2-methoxyfatty acids obtained from marine symbiotic sponges such as the Caribbean *Callyspongia fallax* were discovered to possess antimycobacterial properties (160-161). In a synthetic approach the C_8-C_{14} 2-methoxyfatty acids were prepared and demonstrated activity in the order $C_{10}>C_{12}>C_{14}>C_8$, with 2-methoxydecanoic acid (17) (Figure 3, Table 1) showing an MIC of 40-48 mg/L against the $H_{37}Rv$ strain having an SI above 1.5 (161-162). Interestingly the close relative 2-alkyne fatty acids displayed higher antimycobacterial activity (163). The proposed mechanism of action is inhibition of mycobacterial fatty acid biosynthesis and degradation when toxic levels of the compound are attained, but paradoxically, at non-toxic levels, the alkyne fatty acids may be used for the bacteria to synthesize their mycolic acids. The most active, 2-octadecynoic acid (18) displayed an MIC value of 1.2 mg/L and 2.5 mg/L against *M. smegmatis* mc^2155 and *M. bovis* BCG respectively. Unfortunately there is no mention of the cytotoxicity of the alkyne fatty acids. Other natural fatty acids and lipids have been reported as inhibitors of mycobacterial growth such as the oropheic acids from *Mitrephora glabra* (Annonaceae) (164), or maracin and maracen from the bacterium *Sorangium cellulosum* (165) both with MICs around 12 mg/L but again lacking cytotoxicity data.

From the stem bark of the Vietnamese tree *Micromelum hirsutum* (Rutaceae), the lactone derived from oleic acid, micromolide (19) was isolated and shown to be a potent inhibitor of *M. tuberculosis* growth with an MIC of 1.5 mg/L against the $H_{37}Rv$ strain (166). The selectivity index was calculated to be 63 and it is considered quite a good lead for an unmodified natural product. A report from 2008 published the stereoselective synthesis of the enantiomers of micromolide and several analogue structures with polar groups in the acyclic side chain (167). The data showed that all of the derivatives prepared were less active than the parent compound, indicating that the stereochemistry of the double bond and the lipophilicity of the side chain are the main structural factors contributing to the antimycobacterial activity. The alkenyl unsaturated motif is a particular chemical feature of several natural antimycobacterials suggesting that lipophilic chains of natural products are essential for activity, probably because it allows to diffuse and cross the mycobacterial cell wall.

Another group of important antimycobacterial natural products related to the lipids is the class formed by sterols and triterpenes. The first potent phytochemicals of this class to be identified were the cycloartanes isolated from *Borrichia frutescens* (Asteraceae), an herb occurring in the saline coastal marshes in the USA (168). In that early paper, the MIC of 24,25-epoxycycloartan-3-one (20) was reported to be 8 mg/L against *M. tuberculosis* $H_{37}Rv$, having a selectivity index close to 9 in epithelial kidney cells from the African green monkey (Vero cells). In 1999, two papers describing anti-TB natural products of this class appeared from the same research group (169-170). From the aerial parts of the Kenyan plant *Ajuga remota* (Lamiaceae), ergosterol-5,8-endoperoxide was isolated as a colorless powder showing an MIC of 1 mg/L against the virulent $H_{37}Rv$ strain. Hydroxykulactone, isolated as colorless oil, showed an MIC of 4 mg/L against the same virulent strain. However there is no data regarding the toxicity on mammalian cells and therefore it is difficult to assess whether they have any specificity towards mycobacteria or they are cytotoxic compounds. The Caribbean sea sponge *Svenzea zeai* (Dictyonellidae) contains two *abeo*-sterols named parguesterols which are active against mycobacteria having an MIC value between 8 and 12 mg/L with an SI between 6.5 and 4.3 in relation to Vero cells (171). 3-Epilupeol (21) obtained from the medicinal Chinese flowers of *Chrysanthemum morifolia* (Asteraceae) was found to be a growth inhibitor of the $H_{37}Rv$ strain with an MIC value of 4 mg/L and a SI of 15 against Vero cells (172), constituting a promising scaffold for future development. From the Chilean brown algae *Lessonia nigrescens* (Laminariales), the sterol saringosterol (22) was found to be impressively selective against *M. tuberculosis* having an MIC of 0.125 mg/L for the 24*R* epimer and 1.0 mg/L for the 24*S* epimer, with a very low toxicity ($IC_{50}>128$ mg/L) against Vero cells (173). The calculated SI is above 1024 for the 24*R* epimer and 128 for the 24*S* epimer, evidencing very high mycobacterial specificity for an unmodified natural product and constituting therefore a very interesting lead for further development. Surprisingly after 10 years of the publication of this hit, there are no reports on the evaluation of the compound in a more real setting such as *ex vivo* or *in vivo* models of TB infection. There is no data pertaining to its pharmacodynamic or pharmacokinetic profile or data on its mechanism of action or even the activity of chemical analogues. In order to increase the impact of this hit, several questions still need to be addressed. The stigmastane sterols from *Thallia multiflora* (Maranthaceae) have also been found to be potent and selective antimycobacterials. Stigmast-5-en-3β-ol-7-one, stigmast-4-ene-6β-ol-3-one, stigmast-5,22-dien-3β-ol-7-one (23) and stigmast-4,22-dien-6β-ol-3-one have MIC values between 1 and 4.2 mg/L and showed selectivity indexes above 95 against Vero cells (174). There are other active interesting steroidal natural products such as aegicerin ($H_{37}Rv$ MIC 1.6-3.2 mg/L) isolated from the Peruvian plant *Clavijera procera* (Theophrastaceae) (175) and the steroidal endoperoxides from *Ruprechtia trifolia* (Polygonaceae) (176) both having potent metabolites but lacking cytotoxicity information.

Essential oils are volatile extracts obtained by steam distillation typically from aromatic plants, and they are mainly composed of monoterpenoids (177). The components of essential oils have shown inhibitory effects but these apolar substances also display toxic effects such as phototoxicity, abortive and cancerogenic activities (178-

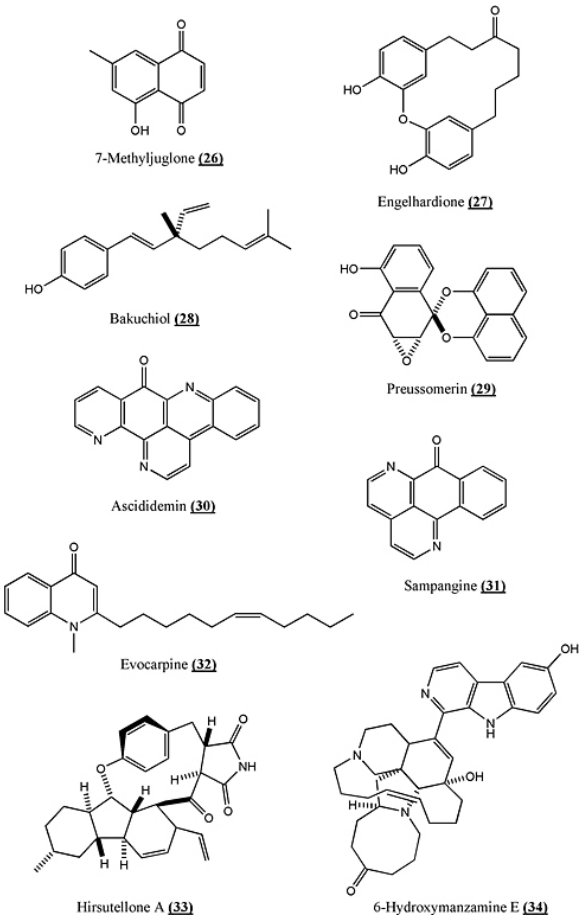

Figure 4. Phenolic and alkaloidal natural products with potent antimycobacterial activity.

180). Among the sesquiterpenoids there are structures with mild activity (MIC~50 mg/L) such as the sesquiterpene lactones costunolide and parthenolide which are also cytotoxic (181-182). The sesquiterpene lactone dehydrocostus lactone (24) from *Saussurea lappa* (Asteraceae) was found to have a significant MIC value around 2 mg/L against *M. tuberculosis* $H_{37}Rv$ (183) but is also cytotoxic with an GIC_{50} between 2.5 and 3.5 mg/L against the cancer line cells HepG2, HeLa and OVCAR-3 (184). 11α-hydroxycinnamosmolide, isolated from the South African plant *Warburgia salutaris* (Canellaceae) showed an MIC value of 40 mg/L against *M. bovis* BCG and inhibited mycobacterial *N*-acetyl-transferase enzyme (185). From the roots of Turkish *Salvia multicaulis* (Lamiaceae), several diterpenoids with antimycobacterial activity have been isolated (186). The quinones 12-demethylmulticauline (25), 2-demethylmultiorthoquionone and 12-methyl-5-dehydroacetylhorminone showed MIC values of 0.46 mg/L, 1.2 mg/L and 0.89 mg/L against the $H_{37}Rv$ strain respectively. However there is no mention about the cytotoxicity of these diterpenoids. From the berries of Turkish *Juniperus excelsa* (Cupressaceae), the diterpenoid sclareol was found to inhibit *M. tuberculosis* $H_{37}Rv$ growth at 6 mg/L (187) however in another paper, it was found to be toxic having the ability to arrest the cell cycle in human breast cancer cells (188).

Several naphthoquinones isolated mainly from *Euclea* species (Ebenaceae) have been found to be inhibitors of *M. tuberculosis* growth (189-190). The activity of 2-hydroxynaphthoquinone (lawsone) has already been discussed earlier in this review. The MIC values of plumbagin, crassiflorone and diospyrone have been determined on *M. tuberculosis* $H_{37}Rv$, being inferior to 5 mg/L (191), however no information of the mammalian cytotoxicity was given. In a report from 2007, lawsone (6), menadione and juglone showed an MIC below or equal to 5 mg/L, but the compounds were cytotoxic against Vero cells, showing a SI below 2 (192). Nonetheless the research team also synthesized several analogue structures finding that 7-methyljuglone (26) (Figure 4, Table 1) displayed a potent 0.5 mg/L MIC against the $H_{37}Rv$ strain and had an SI of around 30. The researchers also found that the naphthoquinones had some affinity for the mycothiol disulfide reductase of *M. tuberculosis*, an enzyme necessary for maintaining the balance of mycothiol, the Actinomycete analogue of glutathione necessary for surviving under exogenous and endogenous oxidative stress (Figure 5) (193-195). Quite a few analogues of plumbagin have been recently synthesized, some of which show promising anti-TB activity but the cytotoxicity information is lacking (196). The lapachones (α and β-pyran naphthoquinones) are interesting antimycobacterial natural products isolated from *Tabebuia* species (Bignoniaceae) and several analogues have been developed with interesting properties but the relative toxicity of these compounds is still unknown (197). Some terpenoid quinones have also been isolated from *Plectranthus* species and they displayed antitubercular activity however the selectivity index was calculated to be below 10 (198).

From the roots of the Asian plant *Engelhardia roxbhurgiana* (Juglandaceae) several secondary metabolites with antitubercular properties were isolated (199). A diphenyl ether ketone named engelhardione (27) has been reported to have an MIC of 0.2 mg/L against the virulent laboratory strain of *M. tuberculosis* but there is no information about its cytotoxicity. Other antimycobacterial metabolites have been obtained from this plant such as 3-methoxyjuglone ($H_{37}Rv$ MIC 0.2 mg/L), 4-hydroxytetralone ($H_{37}Rv$ MIC 4.0 mg/L) and engelharquinone (90-221387 MIC 30 mg/L) but again no information about the specificity was provided (200).

Lipophilic phenols such as 6-paradol and 6-shogaol have shown inhibitory activity against *M. chelonei*, *M. smegmatis*, *M. intracellulare* and *M. xenopi*, having an MIC value between 10 and 15 mg/L but there is no mention about toxicity on mammalian cells (201). Bakuchiol (28) isolated from the seeds of *Psoralea corilifolia* (Papilionaceae) was found to be active against *M. aurum* and *M. bovis* BCG with an MIC of 16 mg/L and 21 mg/L respectively but inactive against *M. smegmatis* showing an MIC above 400 mg/L (202). However in another study the GIC_{50} of this phenol against the human cancer lines AGS and HeLa was 1.5 mg/L and 1.8 mg/L respectively (203).

Phloroglucinol derivatives have also been reported to display inhibition of the growth of mycobacteria. The drummondins D-F isolated from *Hypericum drummondii* (Hypericaceae) showed inhibitory activity towards *M. smegmatis* with MIC values between 1.56 and 3.12 mg/L (204). This class of compound has also been reported to have cytotoxic effects on leukemia and tumoral cell lines with a GIC_{50} in the same order of magnitude (205).

From the lichen fungus *Microsphaeropsis* spp. collected in Thailand, various preussomerins have been isolated and found to inhibit the growth of mycobacteria. Preussomerin (29) was reported to display an MIC value between 1.56 and 3.12 mg/L against *M. tuberculosis* $H_{37}Ra$, having a SI close to 6 (206). Osthrutin is a geranyl coumarin isolated from *Peucedanum ostruthium* (Apiaceae) that has significant antimycobacterial activity against fast growing mycobacteria such as *M. aurum*, *M. smegmatis*, *M. phlei* and *M. fortuitum* (207).

The pentacyclic aromatic pyridoacridone alkaloid, ascididemin (30) is a natural product isolated from marine tunicate *Didemnum* spp (208). The MIC of this compound against *M. tuberculosis* $H_{37}Rv$ is an astonishing 0.1 mg/L however the GIC_{50} on Vero cells is even lower (0.04 mg/L), making the compound less active for *M. tuberculosis* than for mammalian cells (209). This example shows the importance of the toxicity assay when evaluating compounds for growth inhibition against bacteria. An analogue tetraquinone of ascididemin with a dimethylamino-ethenyl chain has a much higher mycobacterial specificity showing an MIC of 0.39 mg/L with a SI of 15 in relation to Vero cells. Using microarray experiments it was found that this compound induces mycobactin biosynthetic genes (210) indicating that the compound interferes with iron uptake (Figure 5) which was confirmed by an increase in the MIC when the mycobacteria was grown in a medium containing an excess of iron, and a decrease of the MIC when the mycobactin gene (*mtbB*) was deleted. The aza-oxoaporphine alkaloid sampangine (31), isolated from the aromatic Ylang-ylang tree *Cananga odorata* (Annonaceae) (211) has been reported to display an MIC value of 0.2 mg/L against *M. intracellulare* (212). The GIC_{50} of sampangine was established to be 4.76 mg/L on Vero cells, having therefore a selectivity index of 23.8 (213). From the unripe fruit of *Evodia rutaecarpa* (Rutaceae) a new class of lipophilic quinolones was reported to have antimycobacterial activity (214). In particular the alkaloid (32) showed an MIC of 2 mg/L against rapid growing *M. smegmatis*, *M. fortuitum* and *M. phlei*. The aporphine alkaloids are also interesting antimycobacterial skeletons, and recently 3-methoxynordomesticine hydrochloride was found to have an MIC value around 5 mg/L against *M. tuberculosis* $H_{37}Rv$ and *M. bovis* BCG (215). The alkaloid inhibited the MurE ligase of *M. tuberculosis* and displayed a selectivity index close to 12 in relation to murine macrophages RAW264.7 cells.

Hirsutellone alkaloids isolated from the pathogenic fungi *Hirsutella nivea* have been found to be potent antimycobacterials (216). Hirsutellone A (33) displayed an MIC of 0.78 mg/L against *M. tuberculosis* $H_{37}Ra$ strain, showing a GIC_{50} higher than 50 mg/L against Vero cell, therefore recording a promising SI above 64. Another interesting alkaloid scaffold is related to the manzamines which have been isolated from the Indonesian sponge *Acanthostrongylophora* sp. The alkaloid 6-hydroxymanzamine E (34) had a potent antitubercular activity with an MIC value of 0.4 mg/L against *M. tuberculosis* $H_{37}Rv$ and a GIC_{50} of 4.3 mg/L on Vero cells, evidencing a selectivity index of 10.75 (217).

6. PERSPECTIVE

Several interesting antimycobacterial hits have been discovered in natural products screening projects such as the saringosterol epimers, micromolide, sampangine and the hirsutellones which share high potency and selectivity towards mycobacteria. These hits should be studied thoroughly for their mechanism of action, for their inhibitory activity on *ex vivo* or *in vivo* infection assays, as well as for structure-activity relationships. Determining their mechanism of action provides a starting point for the elaboration of analogues by rational design, and could also unlock previously neglected antimicrobial molecular targets. It should also be said that chemical biodiversity screening programmes and bioprospection projects should be kept running if we aim to maintain fueling the anti-TB drug pipeline.

Minute amount of bioactive metabolites in their natural sources is often a limitation for detailed biological evaluation. Therefore synthetic procedures should be developed in early stages of lead identification research, as this enables the generation not only of higher amounts of bioactive material but also analogue structures, precursors and derivatives which allow chemical space exploration and identification of essential pharmacophore scaffolds.

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