

A REVIEW OF PLANT-DERIVED COMPOUNDS AND THEIR POTENTIAL  
FOR TREATING COMMUNITY-ASSOCIATED METHICILLIN  
RESISTANT *STAPHYLOCOCCUS AUREUS*

by

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Julia Suzanne Mead

April 2013

## TABLE OF CONTENTS

1. INTRODUCTION .....	1
The History of Methicillin-Resistant <i>Staphylococcus aureus</i> .....	1
Epidemiology .....	3
Transition of MRSA from the Hospital to the Community .....	3
2. COMMUNITY-ASSOCIATED METHICILLIN RESISTANT <i>STAPHYLOCOCCUS AUREUS</i> .....	6
Definition and Genetic Typing of Predominant Strains .....	6
Determinants of Virulence in CA-MRSA Strains .....	10
Clinical Treatment of CA-MRSA Infection .....	11
Increasing Drug Resistance .....	13
3. EXPERIMENTAL AGENTS .....	14
The Call for New Antibiotics .....	14
Novel Antibiotics Currently in Development .....	15
Potential of Natural Products .....	17
Conclusions .....	21
REFERENCES CITED .....	23

## LIST OF FIGURES

Figure	Page
1. Emergence of antibiotic-resistant <i>S. aureus</i> and associated pandemic development .....	3
2. Schematic representation of MRSA strains .....	8
3. Prevalence of penicillin resistance in <i>Staphylococcus aureus</i> in hospitals and in communities, 1940-1972.....	10
4. Systemic antibiotics approved by the US Food and Drug Administration per five year period, 1983-2007.....	16
5. New therapeutic agents for use against MRSA .....	19

## ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) has been a global public health problem, especially in hospital settings, for more than fifty years. Within the last few decades, MRSA has undergone a shift in epidemiology, appearing more frequently in the community, and amongst people without traditional risk factors. Community-acquired (CA) MRSA strains contain a wide range of virulence factors and confer varying drug resistances. Infection with CA-MRSA can often lead to poor clinical outcomes, including death. Current treatments for severe infections are limited, and very few truly novel antibiotics are enrolled in late-phase clinical trials testing by the Food and Drug Administration. Vancomycin is currently the first choice of antibiotic for severe infections, however *S. aureus* strains with intermediate or full resistance to vancomycin have been reported since the early 2000's, thus the need for new antibiotics is urgent. This paper presents a literature review outlining the current body of knowledge regarding the use of plant-derived compounds and their activity against different strains of MRSA. Furthermore, the potential of these compounds for clinical use in treating MRSA infections will be assessed.

## INTRODUCTION

The History of Methicillin Resistant *Staphylococcus aureus*

*Staphylococcus aureus* is a Gram positive cocci commonly found as a commensal organism of the skin, anterior nares and pharynx of human hosts (1). Infection and pathogenesis in humans varies considerably, from self-limiting cases of food poisoning to impetigo to toxic shock syndrome and necrotizing fasciitis (2). *Staphylococcus* was first characterized more than 100 years ago by the surgeon Sir Alexander Ogston when he isolated a culture from a surgical abscess (3,4). It was later named *aureus* for the gold pigmentation produced by its colonies.

*Staphylococcus aureus* exhibits an abundance of virulence factors, some which are encoded for on the chromosome, while others are encoded on extrachromosomal elements, which can be shared with other strains or bacterial species, hence the high frequency of drug resistance (5). Expression of virulence genes is dependent on many factors, thus pathogenesis and clinical manifestations can vary widely. Most frequently, *S. aureus* is responsible for many skin and soft tissue infections (SSTIs), and common toxin-mediated food poisoning (6). Skin infections can vary from mild to severe, causing folliculitis, impetigo, carbuncles, and furunculosis (7). Food poisoning commonly occurs with a rapid onset of symptoms, which usually include nausea, vomiting, and diarrhea of self-limiting duration (6). Further manifestations include such potentially life-threatening conditions such as necrotizing pneumonia, osteomyelitis, and septic arthritis (7). Other life-threatening conditions caused by *S. aureus* infection

include bacteremia, endocarditis, especially when associated with prosthetic valves, sepsis, and toxic shock syndrome (1). Important factors in determining disease progression include myriad predisposing risk factors, (age, immune status, catheterization, drug use, and hospitalization) along with the strain or type of *Staphylococcus* infection acquired (8).

*Staphylococcus* is known for its ability to develop drug resistance quickly (Fig.1); penicillin-resistant *Staphylococcus aureus* (PRSA) was reported shortly after the advent of mass produced penicillin, and was pandemic in the United States through the 1950's and 1960's (9). Penicillin resistance developed due to the acquisition of the  $\beta$ -lactamase gene by *S. aureus*, and was enhanced by antibiotic overuse, which inadvertently selected for bacteria expressing this gene (10). To counter this, semi-synthetic penicillin derivatives were synthesized, such as methicillin, oxacillin, cloxacillin, dicloxacillin, flucloxacillin and nafcillin. Erythromycin, a macrolide antibiotic, was proposed in the early 1950's as another penicillin alternative to treat resistant *Staphylococcus*, however it was withdrawn after less than one year because of rampant acquisition of resistance (11).

Methicillin was first prescribed beginning in 1959 as another alternative to penicillin; two years later Professor Patricia Jevons isolated the first cultures of a methicillin-resistant strain of *Staphylococcus aureus*, now commonly known as MRSA (12).

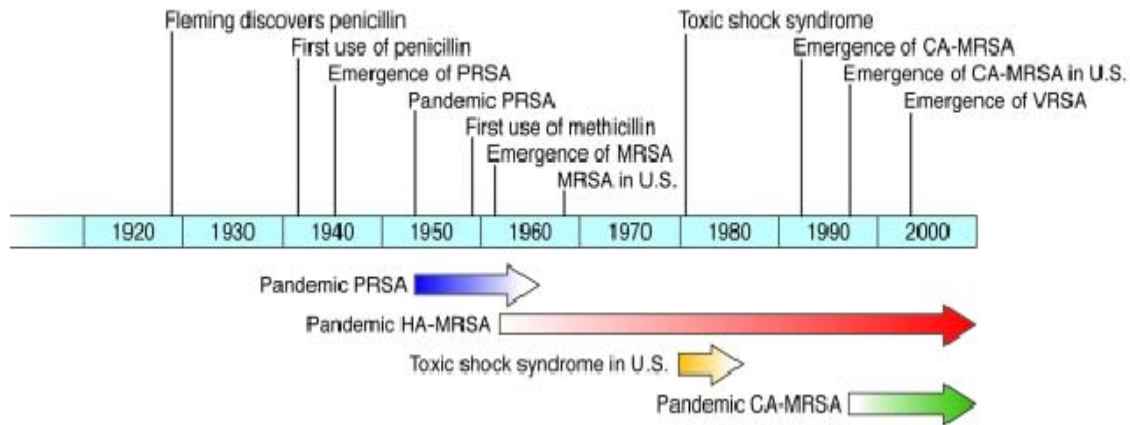


Figure 1. Emergence of antibiotic-resistant *S. aureus* and associated pandemic development. Timeline indicates year resistance developed, or was reported. Arrows indicate length of time in which pandemic occurred. Adapted from (9).

Shortly after the clinical introduction of methicillin, at that time referred to as the “penicillinase-resistant penicillin,” Dr. Jevons cautioned, “It is well known that patients with infected skin can be dangerous sources of infection in hospitals, and the finding of just such a patient infected with a [methicillin] resistant strain in this instance adds an additional warning.” (12).

### Epidemiology

#### Transition of MRSA from the Hospital to the Community

Until the late 1980's, most MRSA infections were nosocomial (denoted Hospital-Associated Methicillin Resistant *Staphylococcus aureus* (HA-MRSA) (13). Hospital acquired infections are still a major health problem- currently, MRSA is endemic in most healthcare and long-term care facilities in the United States and other industrialized

nations (14). Additionally, in the United States, *S. aureus* is the number one cause of hospital-acquired infections, with MRSA accounting for an estimated 60% of isolates recovered from patients admitted to the intensive care unit (9, 14). However, the epidemiology of MRSA has evolved in recent decades with the advent of community-acquired or community-associated strains of MRSA (CA-MRSA), which affect those without traditional risk factors (15). MRSA strains first began appearing in the community in the early 1980's among a population of intravenous drug users living in Detroit, Michigan (16). However, the first true cases of CA-MRSA infections in those with no predisposing risk factors were reported in the early 1990's in rural Australia in individuals with little contact with large medical centers where MRSA is traditionally endemic (17). Further analysis demonstrated that the implicated isolate of MRSA was unique from the other strains circulating Australia, and notably, was not multi-drug resistant (18).

Another turning point away from the paradigm of MRSA as a strictly nosocomial pathogen came in the late 1990's, with the deaths of four otherwise healthy children in Minnesota and North Dakota (19). Each of the children had no traditional risk factors for MRSA infection, such as recent hospitalization or surgery, and died either of sepsis or necrotizing pneumonia caused by infection with *S. aureus* (19).

The increasing incidence of these types of infections, which were occurring in otherwise healthy individuals, often children, raised many questions about whether community-acquired strains were inherently more virulent or more transmissible than other strains. Much of the research over the last decade has sought to monitor the spread

of these now epidemic strains and to elucidate virulence mechanisms which may be novel to community associated MRSA.

COMMUNITY-ASSOCIATED METHICILLIN  
RESISTANT *STAPHYLOCOCCUS AUREUS*

Definition and Genetic Typing of Predominant Strains

The Centers for Infectious Disease Control and Prevention (CDC) define CA-MRSA infection as identification of MRSA in a patient with signs and symptoms of infection with no history of MRSA infection or colonization, no history of admission to a hospital or nursing home during the previous year, and absence of dialysis, surgery, permanent catheters or indwelling medical devices (15). However, definitions of CA-MRSA in the literature vary from study to study, or are not reported at all, and a definitive list of risk factors has not been established (8). Rather, molecular characteristics such as genetic differences between isolates have been useful in distinguishing between HA and CA-MRSA, and among different strains of CA-MRSA.

Since the recognition of CA-MRSA as a distinct clinical entity in 2000, efforts have been made to characterize strains and lineages based on several different methods. Some of the primary methods of molecular typing include pulsed-field gel electrophoresis (PFGE), multi-locus sequence typing (MLST), *spa* typing, and *SCCmec* typing (20). The first three methods rely on sequence-based techniques, while the fourth is based on distinct allotypes of the *SCCmec* element. Different databases exist for each method, often reflecting regional or national nomenclature as well. PFGE is based on distinctions between banding patterns of a digested whole genome, and is known as the gold standard when distinguishing between two closely related strains (20). PFGE types

also denote country of origin (USA 100, etc). MLST is based on genetic variation of seven housekeeping genes, and relies on one database using universal nomenclature (20). *Spa* typing targets polymorphic variable-number tandem repeat regions of staphylococcal protein A (*spa*), and clones predicted by this method usually confer with those found using MLST (20). *SCCmec* classification is based on the type of *SCCmec* element found in a particular isolate, and certain types have been shown to correlate with strains found either in the community or in the hospital (21). The staphylococcal cassette chromosome *mec* (*SCCmec*) is the mobile genetic element responsible for carrying the *mecA* gene, which encodes for an altered penicillin-binding protein (PBP2a) and allows for pan-resistance to  $\beta$ -lactam antibiotics (22).

The first two CA-MRSA strains to be classified were from Western Australia (WA-1) and the United States (MW2), and are both from the MLST sequence type 1 (ST1) (20). Strains closely related to MW2, also known as pulsed-field gel type USA 400, were the most prominent community-associated strains in the United States until 2001, however USA 400 was rapidly replaced by another clone known as USA 300, an ST8 strain (23). USA400 remains as an important pathogen in many regions in North America, however, USA 300 is currently the leading cause of community-associated bacterial infections in the United States (24).

Worldwide, there are thought to be greater than twenty distinct lineages of CA-MRSA, with five strains considered to be predominant (Fig. 2) (20). Much research has been devoted to the identification and origin of the different clones and their global distribution, however, this review will focus primarily on the most prevalent clones in the

United States, USA300 (ST8-IV), which has become the primary cause of skin and soft tissue infections (SSTI) among the general public, along with USA400 (ST1-IV), which is most common in the Pacific Northwest regions of the U.S. (23, 25). However, USA300 is also spreading internationally, and is thought to be the most likely CA-MRSA strain to pose a global epidemic threat (26).

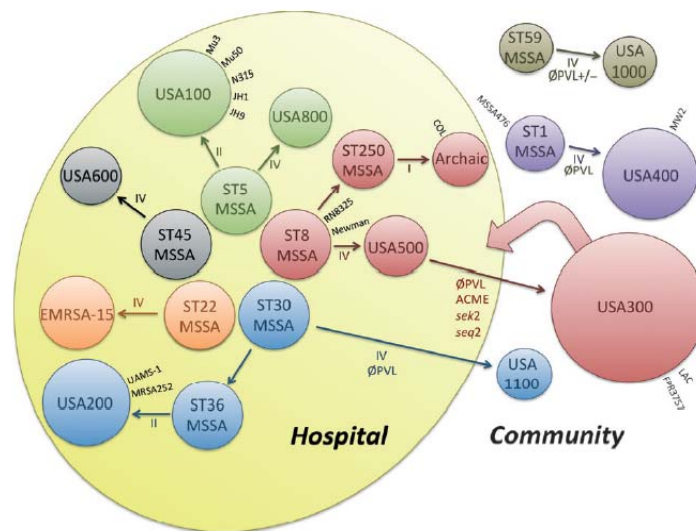


Figure 2. Schematic representation of MRSA strains. Sequence types belonging to established clonal complexes are colored as follows: CC1- purple; CC5- green; CC8- red; CC22- orange; CC30- blue; CC45- black. Roman numerals denote SCCmec type. Commonly used *Staphylococcus aureus* strain names are denoted around their relevant symbol. Adapted from (27).

Initially, one of the most notable characteristics of many of the CA-MRSA strains was their non-multi-drug resistant phenotype, with resistance only to  $\beta$ -lactam antibiotics (28). Historically, resistance to penicillin took longer to develop in community-associated strains than in hospital-acquired strains as well (Fig. 3). One purported reason

for the lack of multi-drug resistance in CA strains is the type of SCC*mec* found in such strains (21). HA-MRSA strains typically include SCC*mec* elements I, II, and III, while several CA-MRSA strains were found to include a novel type, SCC*mec* IV (21).

SCC*mec* IV is much smaller than other elements, thus is thought to be much more mobile (29). Other SCC*mec* cassettes carry genes coding for resistance to various antibiotics, such as erythromycin, spectinomycin, and tobramycin, but are much bulkier and may limit survival outside of hospital settings, which have different selective pressures than the community (21). SCC*mec* IV is thought to be a much more promiscuous element, and has been observed widely among MRSA lineages; additionally it has been demonstrated to show little fitness cost to strains containing the element (29, 30).

Because of this, SCC*mec*IV is thought to have contributed to the success of CA-MRSA strains such as USA300, and their rapid spread through North America (27).

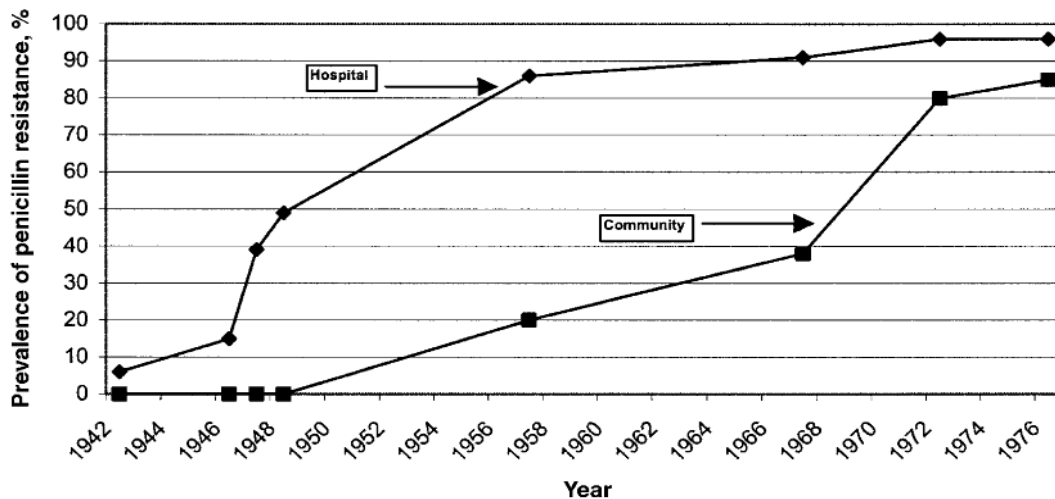


Figure 3. Prevalence of penicillin resistance in *Staphylococcus aureus* in hospitals and in communities, 1940-1972. Penicillin resistance was not reported in community-acquired strains until 1949, when hospital strains were almost 50% resistant. Adapted from (8).

### Determinants of Virulence in CA-MRSA Strains

Virulence factors in CA-MRSA strains are myriad, with both secreted and surface-bound factors having an impact. Community-associated strains have been found to express modified levels of virulence genes when compared to hospital-acquired strains, and have shown increased capacity for host immune evasion (31, 32). Toxins which are characteristic of *S. aureus*, but which aren't present in all isolates include cytotoxins,  $\alpha$ -hemolysin (*hla*), several enterotoxins and the exfoliative toxin (1). One such toxin, which has been the source of heated debate, is known as the Panton-Valentine leukocidin (PVL) leukotoxin, which has been epidemiologically linked to CA-MRSA strains of interest (33). PVL is a pore-forming toxin which induces necrosis and apoptosis of neutrophils (34). Numerous studies have sought to elucidate the exact role of PVL in the pathogenesis of certain strains, and its contribution to virulence, however a definitive role has not been established (9).

Other unique toxins include enterotoxin K and Q, which are encoded on a genetic island, SaP13, and are found in USA300 strains but not USA400 strains and may explain differences in pathogenicity between the two strains (35). Another genetic element acquired only by USA300 strains is the arginine catabolic mobile element (ACME) which is hypothesized to contribute to colonization of the bacteria, perhaps by modifying the host skin environment (36).

A specific mechanism of virulence utilized by CA-MRSA is the differential regulation of virulence genes. For example, USA300 clones have demonstrated increased expression of the gene *hla*, when compared with other *S. aureus* isolates (31,

37). *Hla* codes for  $\alpha$ -hemolysin, a pore forming toxin which results in rapid cell lysis, destroying a variety of host cells (38). Inhibiting the expression of toxin-encoding genes such as *hla* has been an area of active research in the study of novel treatments for MRSA infections, in order to minimize the deleterious effects (39). *Hla* expression was also reduced when genes located on the *S. aureus* exoprotein expression (*Sae*) locus were deleted, illustrating the importance of gene regulation in the pathogenicity of strains such as USA300 (40). The *Sae* locus contains four genes, two of which make up the *SaeR/S* two-component regulatory system, which is activated by different environmental conditions and is thought to sense changes in cell envelope integrity, however the specific ligand for *SaeS* is unknown (41). Typically, two-component regulatory systems function by relaying an environmental signal from a membrane-bound receptor to a response regulator, which can mediate the transcription of target genes (40). When *SaeR* is in an active state, several virulence genes are induced, including *hla* and *lukS-PV*, which encodes for a subunit of PVL (40). In experiments using *Sae* knockout strains of USA300, virulence was attenuated in several murine models of infection, including sepsis and pneumonia, further demonstrating the impact of gene regulation on virulence and clinical outcomes (31, 40, 42, 43).

#### Clinical Treatment of CA-MRSA Infection

Treatment of *S. aureus* infections is a major public health challenge due to variance in drug resistance between strains, and a wide range of clinical presentations, which span from simple to life-threatening. Random mutation and plasmid-mediated horizontal gene transfer have introduced drug resistance capabilities to ever-increasing

types of antibiotics, even in community associated strains which were previously described as being less drug-resistant (2). As strains such as USA300 have continued to circulate more widely, certain isolates have acquired a variety of plasmids conferring additional drug resistance, in some cases comparable to hospital-associated strains (44). Though antibiotic susceptibilities may differ from strain to strain, empiric therapy is often used until cultures can be obtained and tested for resistance (45). Current recommended treatment for common CA-MRSA infections, such as simple SSTIs, calls for incision and drainage of wound unless presented with co-morbidities or risk factors, in which case oral treatments include clindamycin, long-acting tetracyclines such as doxycycline and minocycline, and trimethoprim-sulfamethoxazole (TMP-SMX) (46). Rifampin or fusidic acid can be used as treatment, but only as an adjunct because of the high probability of resistance developing when used alone (47). More severe MRSA infections can have very limited treatment options.

Vancomycin is currently the first choice for intravenous treatment of complicated MRSA infection, however, treatment can be deleterious to patients, treatment failure rates are relatively high, and the widespread emergence of vancomycin-resistant *S. aureus* strains is a very real fear (48,49,50). Alternative antibiotics, such as daptomycin, tigecycline, and linezolid can also be prescribed for more severe MRSA infections such as bacteremia or pneumonia, but have not proved any more efficacious than vancomycin in clinical trials, and have similar toxicities (46, 51, 52). Thus options remain limited for treatment, and even more so in strains which do not respond to vancomycin.

Increasing Drug Resistance: Initially, Vancomycin was not widely used after being introduced, with less toxic but similarly efficacious antibiotics being favored instead (53). However, beginning in the early 1980's its use became much more widespread, first as an oral treatment for pseudomembranous enterocolitis caused by *Clostridium difficile*, and secondly to treat MRSA, which was becoming more common outside of hospital settings (54). An unfortunate consequence of this increased usage was the dawn of vancomycin-intermediate *S. aureus* strains (VISA) in Japanese isolates in 1997 (55). VISA isolates were recorded shortly afterward in the United States, with minimum inhibitory concentrations (MICs) of vancomycin ranging from 4-8 µg/mL (56). VISA can emerge when certain strains of *S. aureus* produce an abundance of non-cross-linked-D-alanyl D-alanine, which can trap vancomycin and prevent effective dissemination to the target area (57). Unfortunately, another more potent mechanism of vancomycin resistance has emerged, albeit on a scale that has been limited to thirteen cases in the U.S. thus far (56). This resistance is thought to have been transferred by a plasmid from a vancomycin-resistant enterococci (VRE), and was first isolated in Michigan in 2002 (53). Vancomycin resistance is conferred on the *vanA* gene, which alters peptidoglycan intermediates targeted by vancomycin, and reduces the binding affinity of the drug (53). In this case, the MIC for vancomycin was  $\geq 32$  µg/mL (53). Interestingly, in the first two cases of VRSA diagnosed in the U.S., both patients were found to be co-colonized with VRE and VRSA, giving credence to the unfortunate hypothesis that horizontal gene transfer between these two species was increasing drug resistance (56, 58, 59).

## EXPERIMENTAL AGENTS

The Call for New Antibiotics

Given the increase in single and multi-drug resistance of a variety of pathogens, such as *Mycobacterium tuberculosis*, *Streptotoccus pneumoniae*, gram-negative bacilli, and *S. aureus*, the development and approval of novel antibacterial agents is critical to public health in the coming decades (60). This fact, coupled with the stagnation in the creation and usage of novel antibiotic agents and classes since the early 1990's (Fig. 4), illustrates the clear need for new treatments for these so-called “superbugs,” before current options become any more limited (60). Currently, there are several new antibiotics in developmental stages which are showing promise in combating MRSA. Another exciting branch of research is focused on natural products, derived mainly from plants, which have shown antimicrobial effects and MICs comparable to many antibiotics currently on the market (39, 79).

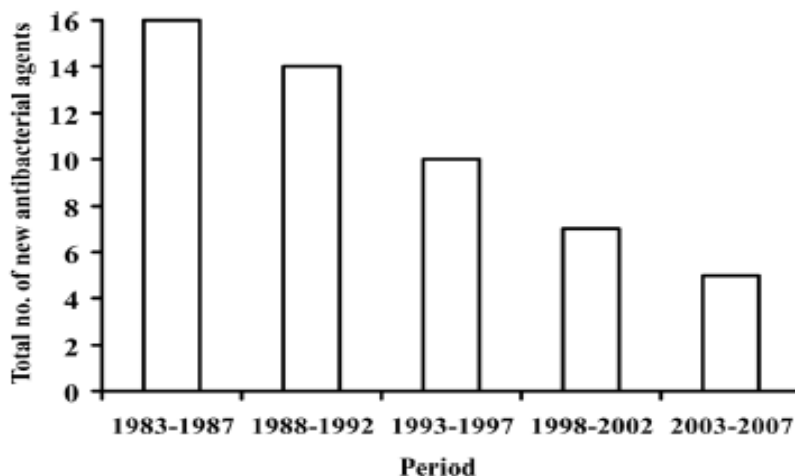


Figure 4. Systemic antibiotics approved by the US Food and Drug Administration per five year period, 1983-2007. Adapted from (60)

### Novel Antibiotics Currently in Development

There are many new antibiotics in development, some of which are undergoing phase II and III clinical trials for FDA approval. Several candidates are simply new members of existing antibacterial classes (Fig. 5) (61). For example, Ceftobiprole and ceftaroline are new generation cephalosporins with strong affinity for PBP2a (62). Ceftobiprole has also showed stability to  $\beta$ -lactamases (63). A recent study by Saleh-Mgir et al. demonstrated the efficacy of ceftobiprole against osteomyelitis infection in rabbits, induced with several CA-MRSA clones, including USA300. *In vivo* results showed decreased bacterial loads in the bones of rabbits treated with ceftobiprole compared to those treated with vancomycin (64). Another study compared the efficacy of ceftaroline and vancomycin in another rabbit model of acute osteomyelitis, and demonstrated similar results, with the new generation cephalosporins performing better than vancomycin (65). Ceftobiprole was initially approved for use by the FDA, but has since been discontinued in the United States until further clinical trials are performed (66). Ceftaroline has been approved for use in community-associated bacterial pneumonia caused by MSSA, and acute SSTIs caused by both MRSA and MSSA isolates (67).

Dalbavancin and oritovancin are semi-synthetic lipoglycopeptides derivatives, with structural similarity to vancomycin. Both of these agents inhibit peptidoglycan synthesis; additionally, these agents disrupt cell membrane integrity through membrane depolarization (68). It has also been determined that their half-lives are longer when compared with vancomycin and teicoplanin, allowing for less frequent dosing (68). Both

dalbavancin and oritovancin are in phase III clinical trials, but neither has received FDA approval (69, 70)

Iclaprim is another antibiotic awaiting FDA approval, which acts as a dihydrofolate reductase (DHFR) inhibitor, disrupting the synthesis of folate (71). This drug is structurally similar to trimethoprim, and thus has been shown to act in a synergistic manner in combination with sulfonamides, analogous to the commonly prescribed combination of trimethoprim-sulfamethoxazole (71). Iclaprim is still undergoing phase III clinical trials, to assess the safety of oral administration as well as intravenous use (71).

RX-1741, also known as radezolid, is a second generation oxazolidinone which is currently in clinical trials (72). Oxazolidinones are a relatively new class of antibiotics which inhibit protein synthesis; linezolid is the only one currently licensed for clinical use (72). Though resistance to linezolid in the clinic is not common, it has been documented, thus further drugs within this class are in development (72).

Clearly, major drug companies favor the development of novel versions of existing antibiotic classes, because often their safety and efficacy has already been established through previous *in vivo* testing. However, given the history of *S. aureus* and its proclivity to develop resistance quickly, and to many distinct classes of antibiotics, completely novel mechanisms or new classes hold promise in staving off further drug resistance. (12, 55, 61). Regardless, several of the aforementioned compounds have shown efficacy, coupled with low toxicity, in clinical trials and will likely be approved by the FDA in the coming years for treatment for a variety of MRSA infections (61).

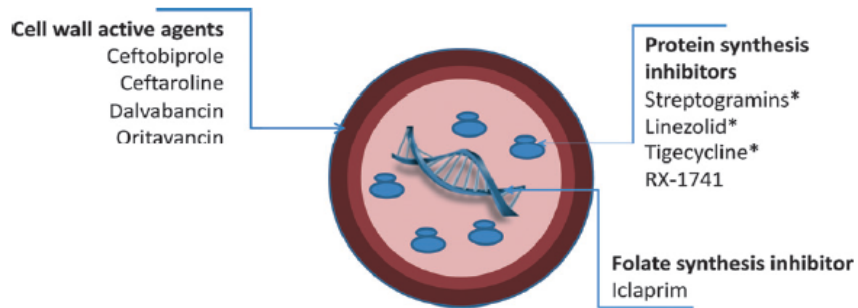


Figure 5. New therapeutic agents for use against MRSA. Those currently in clinical use are denoted with (\*), others are agents still in development. Adapted from (61).

### The Potential of Natural Products

A variety of plant-derived natural products have been shown to demonstrate antibacterial effects, hence the use of many plant-based treatments in traditional medicine throughout history (73). The modes of action observed by these compounds range from purely bactericidal effects, to modification of virulence mechanisms or modulation of drug resistance profiles (39, 74, 75).

The lab of Tsutomu Hatano of Okayama University has researched the effects of a variety of plant derived components on methicillin susceptible and methicillin resistant strains of *Staphylococcus aureus*, along with a variety of other pathogenic organisms, such as vancomycin resistant enterococci, *Helicobacter pylori*, and *Pseudomonas aeruginosa*. Several of the compounds studied by this lab were found to have antibacterial effects on MRSA, with minimum inhibitory concentrations (MICs) ranging from 60 µg/ml in theasinensin A, a polyphenol compound derived from the *Camellia sinensis* plant, to less potent extracts such as geraniol, a monoterpene constituent isolated from *Zanthoxylum* spp. fruit with an MIC of 512 µg/ml (75). While many of the

compounds tested did not exhibit inherent bactericidal properties, several compounds effectively suppressed the antibiotic resistance of different MRSA strains, making the bacteria more susceptible to either oxacillin or penicillin. Results such as these are promising therapeutically in their potential to allow for reduction in antibiotic dosage and diminish potential side effects (76).

Seven distinct lignans were isolated from *Larrea tridentata*, a plant used in Mexican traditional medicine, with one in particular, demethoxy-6-Odemethylisoguaiacin, showing activity against a MRSA strain and a MSSA strain, along with three clinical isolates of *S. aureus*, with MICs ~ 25 µg/ml (77). A medicinal plant from Thailand, *Garcinia cowa*, contains many antibacterial compounds in its stem bark, several of which were tested against a variety of pathogenic bacteria (78). Five compounds were active against a MRSA strain, with MIC values ranging from 2-16 µg/mL, respectively (78).

In the last several years, there has been an upswing in publications demonstrating the efficacy of various plant derived products which have gone beyond simply determining MIC values, and have begun to explore the effects of these compounds on bacterial growth rate (79), minimal bactericidal concentration (MBC) (80), or putative modes of action (81). These and other tests can help to determine whether a particular compound may be a candidate to treat a specific condition, such as an infection of an indwelling medical device (81).

*Achyrocline satureioides*, a South American medicinal plant, has been studied for use in a variety of biomedical research, with one compound, achyrofuran, showing

particularly high bactericidal activity (79). This paper is unique in that the activity of the compound was tested on a MSSA strain, a vancomycin-intermediate *Staphylococcus aureus* strain (VISA), and over twenty clinical isolates of *S. aureus* with differing antibiotic susceptibilities, including USA300 (79). Achyrofuran was found to have an extremely low MIC value, of 0.25 µg/ml for the MSSA strain and 0.12 µg/ml for the VISA strain (79). Time-kill experiments also showed promising results; MSSA and VISA were incubated with one to four times the MIC of achyrofuran and all bacteria was killed within twelve hours. Growth was also inhibited in all twenty-two of the clinical isolates of MRSA (79). Other experiments determined achyrofuran acts by increasing cell permeability, which was determined by measuring uptake of a fluorescent stain when cells were treated with the compound (79). These promising results should encourage further study into the toxicity and pharmacokinetics of achyrofuran, and its potential use as a treatment for VISA infections (79).

A study by Nowakowska et al. sought to evaluate the therapeutic effects of 8-hydroxyserrulat-14-en-19-oic acid (EN4), a compound extracted from the leaves of *Eremophila neglecta*, which may have potential in treating implant-associated infections (IAI), which can be especially difficult to treat (81). The study looked at a variety of organisms, including a strain of MSSA, SA113, which is known to produce a polysaccharide intercellular adhesin (PIA)-mediated biofilm, which can contribute to bacterial persistence and resistance to treatment (81). MIC of EN4 during logarithmic growth was 25 µg/ml for a strain of MRSA, MSSA, and the MSSA SA113 isolate (81). A time-kill assay was also performed with concentrations of EN4 ranging from half to four

times the MIC, with bactericidal effects observed within five minutes at the MBC (50 µg/ml) (81). To assess whether EN4 might be effective against bacteria adhering to a surface, several experiments studied bacterial growth in the stationary phase, to mimic growth conditions in a biofilm environment. MBCs were comparable to those measured for MIC during logarithmic growth. However, two *in vivo* tests showed serious limitations of EN4, both in a tissue cage mouse model, and in interaction with physiological fluids and proteins. EN4 failed to exhibit antibacterial properties, prevent bacterial adhesion to tissue cages or clear infection in the mouse model, and cytotoxic effects were found to be completely inhibited when exposed to 10% fetal bovine serum. These results clearly illustrate the necessity to perform *in vivo* testing in early stages of research, before broad claims can be made about whether a compound may have potential as a realistic antibiotic candidate.

A paper of note, published by Long, et al. in 2013, focused on the effects of a licorice root (*Glycyrrhiza* spp.) extract component, 18β-glycyrrhetic acid (GRA). Glycyrrhizic acid (GA) is a triterpenoid saponin isolated from the licorice plant, which is hydrolyzed to GRA once metabolized by gut bacteria (82). Previous studies have shown antiviral and antibacterial effects of GRA, however this study sought to demonstrate the bactericidal activity of GRA, *in vivo* and *in vitro*, and the ability of GRA to modulate virulence gene expression in MRSA, specifically PFGE type USA 300 (76,83). GRA was shown to attenuate survival of MRSA *in vitro*, with a minimum inhibitory concentration (MIC) of 60µg/ml. *In vivo* experiments demonstrated that treatment with GRA reduced abscess size in mouse models of staphylococcal skin infections, and

modulated host response to infection. GRA also down-regulated the gene expression of several key virulence factors, both *in vitro* and *in vivo*, such as the alpha-toxin gene, *hla*, and *saeR*, the regulatory gene component of the *SaeR/S* regulatory system. Further studies are needed to determine whether resistance to GRA could be induced, and to elucidate mechanisms of action, both in the bacteria and the human host (39). However, this study is a thorough analysis of a specific compound, with relevant *in vivo* studies and gene expression data to further support basic measures such as MIC values.

Papers such as these highlight the need for more in-depth and pertinent studies of promising compounds, which are clearly abundant, such as determining mammalian toxicity levels, pharmacokinetics, and molecular mechanisms of action. Without further study, plant derived natural products cannot move closer to being used clinically, or provide novel treatment options for increasingly drug resistant pathogens.

### Conclusions

From the dawn of the antibiotic age, with the discovery and introduction of the sulfonamides and penicillin in the late 1930's and 1940's, drug resistance by bacterial species has followed closely behind (60). Coupled with the sharp decline in novel antibiotic approval in the United States, the so-called golden age is well behind us (11, 84). Rather, a new age of pathogenic bacteria is upon us, with each species and strain exhibiting a distinct repertoire of single, multiple or total drug resistances. Undoubtedly, solutions to help mitigate this alarming trend are myriad, and include increased screening and surveillance, infection control measures, and changes in antibiotic dispensation

practices by doctors (60, 85). Furthermore, the future calls for more, truly novel antibiotics, not simply derivatives of antibiotic classes to which bacteria have already shown resistance (61). Several promising candidates exist, however, research and development of novel drugs must be made a national priority, which will require the cooperation of scientific researchers, pharmaceutical companies, governmental regulatory agencies, doctors, and the general public (86). Our shared livelihood may depend on it.

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