Coevolution: Mankind and Microbes[⊥]

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In just 70 short years mankind has progressed from euphoria to despair over the effectiveness of antibiotics to protect and to cure mankind from morbidity and mortality from infectious diseases. Resistance due to evolutionary factors was observed shortly after antibiotics came into use and is now not only widespread but appears to be inevitable. This review is a rather personalized account of the various attempts to deal with this problem over time.

Introduction

Mankind and microbes have coexisted in competitive equilibrium since long before recorded history. Every part of the body in contact with the environment possesses a thriving population of bacteria that normally cause no harm. In fact, about 100 thousand billion bacteria live in or on the average adult human.[1] One gram of feces contains about 100 million aerobic bacteria as well as 100 trillion anaerobic colony-forming units![2] Despite this normal coexistence, humans are always potentially susceptible to morbidity or death from infections when this equilibrium is disturbed. This is particularly true for the very young and the very old. Occasionally throughout history there have been periodic pandemics that decimated mankind by mechanisms that were previously completely unknown. The serial outbreaks of bubonic plague of the Middle Ages provide a well-known example. Mankind has been essentially helpless in the face of these disasters until quite recent times. The search for chemical cures initially involved empirical searches involving *Materia Medica*, basically pharmacognosy. Although, as will be seen below, higher plants are now known to elaborate many antimicrobial agents and some important classic anti-infective drugs such as quinine and emetine have emerged from this effort, no great progress was made against bacterial pathogens. The tide began to turn in mankind's favor about 150 years ago. The institution of public health measures (quarantine, pasteurization of milk, chlorination of water supplies, vaccination, avoidance of high-risk behaviors, etc.) significantly decreased the toll on humanity, particularly in the 20th century. The advent of antimicrobial drugs greatly accelerated this trend.

Putatively, the discovery of antimicrobial agents that can be used systemically is the most important medical event in the 20th century. The story of the discovery by Sir Alexander Fleming of the penicillins in 1929 is well-known.[3] Likewise, equally famous is the discovery of the oral activity of sulfonamides in the 1930s by Domagk and Bovet.[4] The discovery by Dubos of gramicidin in 1939[5] and the introduction of penicillin into clinical trials in 1941 as a consequence of the work of Chain, Heatly, and Florey[6] were followed in short order by the discovery of a plethora of penicillins and cephalosporins, streptomycin,[7] chloramphenicol,[8] tetracycline,[9] and erythromycin.[10] During this time the discovery of useful anti-infectives shifted largely from the synthetic laboratory to a search among natural products. The 1940s and 1950s remain the glory days of antibiotic discovery. Public euphoria led to the common use of the appellation "miracle drugs", and several Nobel prizes were awarded to their discoverers.

Unfortunately, shortly after the clinical introduction of antibiotics ominous reports of microbial resistance during the course of treatment began to appear in the literature. Penicillin was first used in 1941, and by 1942 publications relating to resistance appeared.[11] Resistance was noted with increasing frequency until this phenomenon could no longer be ignored. By 1945 the incidence of penicillin-resistant *Staphylococcus aureus* infections had already reached 14%.[12] Methicillin was introduced in 1960 to deal with this,[13] and very soon after its introduction, clinical resistance was noted.[14] By 1968 methicillin-resistant *Staphylococcus aureus* (MRSA) infections were noted to be epidemic in some hospitals and were appearing in the general population as well.[15] In 1970 interspecies transfer of multiple genes for resistance to several unrelated families of antibiotics was noted in Japan and very soon thereafter virtually everywhere.[16] Today some experts predict a return to the comparatively tragic and defenseless preantibiotic days. Significant clinical resistance is now known for virtually all antibiotics in medical use. Unfortunately, for commercial reasons, the problem is getting worse since big pharma has largely withdrawn from research directed toward new antibiotic discovery.[17]

The following is a somewhat personalized account of these turbulent and exciting times along with an account of some of the avenues pursued, the lessons learned, and a cautious prediction of the road ahead.

The Resistance Phenomenon

Resistance represents a decreased responsiveness to antimicrobials and can be either slight or profound. Resistance is one of the mechanisms that bacteria use to enable them to survive in the competitive world that they inhabit and arguably has existed as long as have bacteria themselves. Resistance can be divided into two classes: intrinsic and acquired. Intrinsic resistance occurs naturally since no antimicrobial agent kills or inhibits all treatment-naïve microbes. Acquired resistance occurs following encounter with anti-infectives. The acquired genes for resistance can be obtained by transfer from other microbes (horizontal transmission) or by mutation of their own genes (vertical transmission).

It is clear by now that bacterial resistance to chemotherapy is not new, unexpected, or avoidable. Bacteria are superbly efficient evolutionary machines able to receive, modify, and consolidate genes from a remarkable range of donors.

Bacteria can share their genes using transposons, conjugation, transduction, transformation, and transposition.[1] These processes are illustrated in Figure 1.[18] Thus, they are remarkably versatile genetically and can share their genes many times within their brief lifetimes with the result that bacteria can exist, even thrive, in remarkably hostile environments. Only a few cells need to survive and reproduce to change the genetic character of a population of bacteria.

A simple experiment illustrating this is summarized in Figure 2.[19] When a culture of *Staphylococcus aureus* is allowed to grow

[⊥] Dedicated to Dr. G. Robert Pettit of Arizona State University for his pioneering work on bioactive natural products. Adapted from the Norman R. Farnsworth Research Achievement Award address, 48th Annual Meeting of the American Society for Pharmacognosy, Portland, Maine, July 14–18, 2007.

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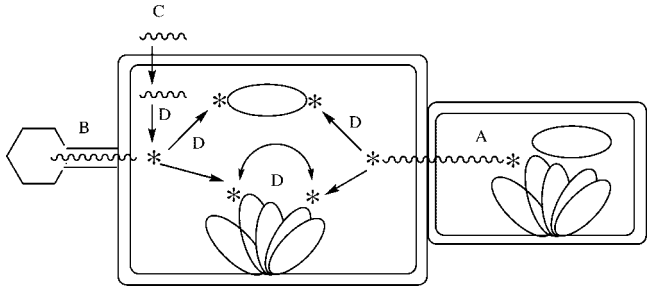


Figure 1. Schematic illustration of the means by which bacteria acquire and transfer antibiotic resistance genes. * = Transposon (mobile resistance gene segments capable of inserting into other genes. A = Conjugation. Intra- or interspecies transfer of duplicate plasmids by cellular contact. B = Transduction. Introduction of gene segments into a target cell by a virus. C = Transformation. Uptake into a cell of foreign gene segments. D = Transposition. Movement of a gene segment to a new position or gene.

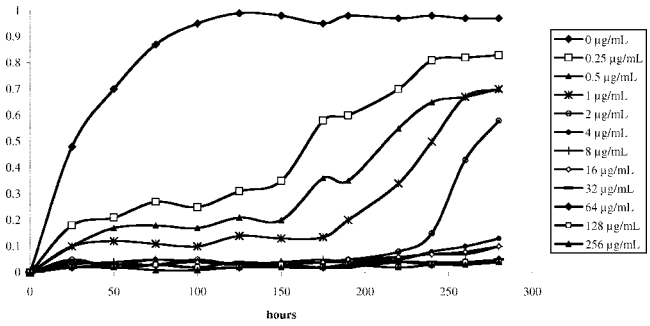


Figure 2. Growth of *Staphylococcus aureus* ATCC 13709 over a 14 day period in the presence and absence of increasing amounts of doxycycline hydrochloride.[19]

unimpeded on nutrient agar, it grows logarithmically and in about 100 h it has ceased to grow further, having exhausted the medium's capacity to support further growth. The addition of increasing quantities of doxycycline in such experiments results, at quite low concentrations, in a delay in growth that becomes more pronounced as the concentration increases. Soon a concentration is reached at which no growth is observed for many hours. If the concentration is high enough, then no growth is observed throughout this long experiment (this experiment proceeded for 12.5 days, whereas the normal time for measuring antibiosis is 24 h). Note however that at a slightly lesser concentration growth eventually occurs. If one cultures cells from this breakthrough culture, a high degree of initial resistance is observed. Similar data are obtained with virtually all presently used antimicrobials.

The implications of this are profound. If an antibiotic is to be used, it should be applied vigorously, continuously, and in sufficient concentration that resistance cannot develop.

Another important factor associated with resistance is the rapidity with which bacteria reproduce. Under ideal conditions many common bacteria can double their numbers about every 20–30 min by binary fission. This is several thousands of times faster than mankind. If allowed to reproduce at this rate for just 24 h, more than a trillion cells could result. Fortunately this almost never happens outside of the laboratory, as the actual doubling rate under normal environmental conditions is usually dramatically less. Mankind, on the other hand, could produce a maximum of 280 children in a century if the original pair and all the progeny participated fully from age 15 through menopause and one presumes equal numbers of males and females. Bacteria can produce this many individuals in just four hours. This growth rate advantage makes it possible for bacteria to adjust much more rapidly to threatening changes in their environment than can mankind.

Thus, in retrospect, it is not too surprising that bacteria adapted in only a few years to the environmental disaster that antibiotics represented to them. The loss of individual bacteria to chemotherapy has been staggering, but the few initial survivors that were able to resist multiplied rapidly so that their population as a whole evolved into resistance in response to the selecting pressure of antimicrobial agents. This rationalizes the present situation.

The general stratagems that bacteria employ to resist antibiotics fall into a few main types. These are inactivation of antibiotics through enzymatic attack, enhanced production of target enzymes, self-modification of essential pathways and structures, decreases in uptake and increases in expulsion of drugs, and the like. The diversity of these modes makes finding a universal solution to the resistance problem extremely difficult.

Mankind adapts dramatically more slowly to the advent of new pathogens, but one believes that in time we will also adapt to this situation as our gene pool is "refined". In the meanwhile introduction of novel antimicrobial measures and better use of those we presently have is our best move.

The following outlines what chemists have done in this area and indicates some of what might yet be done. At first employed individually, these stratagems are increasingly being used in combinations. In evaluating these, it is important to bear in mind that resistance is usually not an all or none proposition. Any increase in the dose needed in order to be effective represents resistance. One consequence is that an increase in potency of a drug may be sufficient to control a given resistant population. This stratagem is, of course, limited to cases where blood and tissue levels can be increased safely to the required level.

A Brief Historical Context

The main effective strategies employed to date to deal with the resistance problem are relatively few. These include searching for novel antibiotics, modification of existing antibiotics, synthesis and directed biosynthesis of novel antimicrobials, inhibition of bacterial enzymes that inactivate antibiotics, interference with drug export mechanisms of bacteria, employment of potentiators of antibiotic action, rapid molecular diagnostics, use of combinations of antibiotics, employment of immunostimulants, use of probiotics, administration of antimutagenic agents, co-infection, and trolling the genome to identify new targets for antibiotics.

To deal with infectious diseases in the 1800s, the choices were dramatically fewer. The major advances were the recognition of the role that bacteria and viruses played in communicable diseases in the first place, purification of water supplies, pasteurization, vaccination and quarantine, the use of external disinfectants, and the like. These decreased mortality significantly and were increasingly put into play in the early 1900s. They were, however, woefully inadequate to deal with infection of battlefield wounds in World War I and to address the disaster of the great influenza outbreak of 1917–1918. The latter caused more deaths than all the slaughter of that Great War.

The data in Figure 3 are instructive. The significant decrease in mortality from infectious diseases in the United States from 1900 to the mid-1930s is primarily due to the institution of public health measures. The introduction of sulfonamides and later of antibiotics continued the downward trend. Undoubtedly the leveling off of the mortality rates that occurred in the mid-1950s would have occurred at a higher level were antimicrobials not available. The upward trend that took place about 1980 can be attributed in part to AIDS and in part to the widespread emergence of resistance of bacteria to chemotherapy.

For those with a taste for irony, sulfanilamide was synthesized in 1908[22] but was not found to have antimicrobial properties until the 1930s. One can only imagine the suffering and deaths that could have been averted if this had been done sooner. One of the consequences is that firms active in antimicrobial research routinely

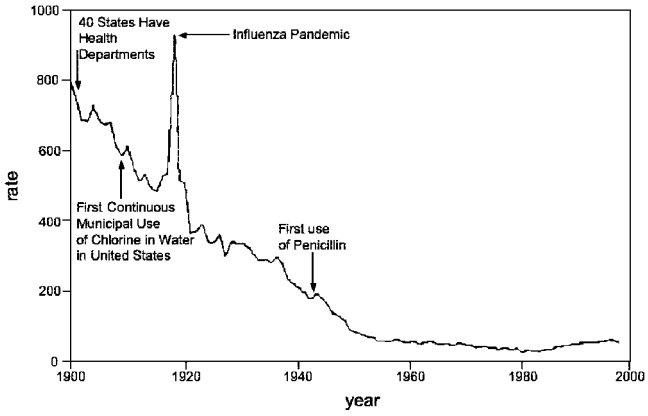


Figure 3. Death rates per 100 00 population per year from infectious diseases in the United States from 1990 to 1996.[20,21]

examine to this day all available substances for activity in all available tests so as not to miss something important again.

At the dawn of 1900, the three leading causes of death in the United States were tuberculosis, pneumonia, and enteritis/diarrhea. If one adds diphtheria, they collectively caused one-third of all deaths and led all other causes.[20,21] The fatality rate before antibiotics due to *Staphylococcus aureus* septicemia was 90%. Life expectancy was limited to 50–60 years largely due to these causes.[1] A simple sneeze led to the reflexive issuance of a brief oral blessing for fear that this was the first sign of imminent fatality! This custom persists worldwide despite the alteration in outlook that effective antibiosis has wrought.

The discovery of the antibacterial sulfonamides in the 1930s and of one after another major antibiotic in the 1940–1950 era dramatically changed this picture, with, for example, the mortality from staphylococcal septicemia plummeting to about 25%.[23] Even greater decreases in mortality from otherwise highly fatal diseases ushered in the age of the miracle drugs, and the discoverers became popular heroes.

Carried away by popular and medical euphoria these drugs were not employed in retrospect with appropriate caution, so that resistance to their use began to be noticed. Although regarded at first as a curious nuisance, before long the incidence of bacterial resistance during therapy became alarming. There is no question that a precious feature of our life has been significantly squandered due to careless application of these drugs.[1] How did this happen, what has been tried, with what result, and what can we presently do about it?

The Discovery Phase

Whereas the first antibiotics were discovered by individuals pursuing their curiosities in university settings with minimal funds and facilities (penicillins, tyrothricin, streptomycin), the economic value of their work was quickly recognized and industrial laboratories quickly organized and financed teams of specialists and set definite goals for their work. Thus the nature of antibiotic research passed quickly from a cottage industry mode into an industrial mode, where it largely remains today.

Consequently, chloramphenicol, tetracyclines, erythromycin, glycopeptides like vancomycin, and rifamycin (all natural products) were introduced into medical practice in rapid order. A great many other agents were found by systematic screening of soil microbes and explored for a time, but most were found to be too toxic, too narrow in spectrum, or ineffective in curing mice with experimental infections for development and became literature curiosities (streptothricin comes to mind as an example).[24]

The multitude of known substances created problems for dereplication. In many cases it could take a year or more of effort to purify, identify, and evaluate a lead substance. On those occasions when the product turned out to be previously known, a great deal of effort had thus come to naught. To avoid this, elaborate schemes involving chromatography, use of resistant organisms, presumed physicochemical behavior, and the like were employed.[25,26] Many of these are truly ingenious but of such great proprietary value that they were almost never published then or now.

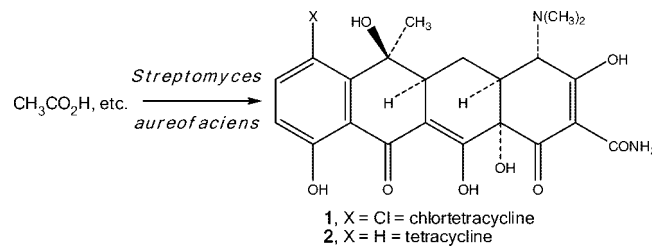
Whereas the failed agents were useful for the producing microorganisms, they were not suited for human use. Obviously the streptomycetes do not concern themselves with matters of mammalian toxicity, blood levels, excretion rates, and the like when they elaborate antibiotics. Even the useful ones have deficiencies that can often be remedied by artful chemical transformation of the natural products. For example, penicillin G was of outstanding utility but was relatively unstable and narrow in spectrum. Alteration of the C-7 side chain produced penicillin V, which had a narrower antibiotic spectrum but was more effective orally due to its enhanced stability against acid degradation. Methicillin was broader in some aspects of spectrum, being stable to the classical penicillinases. Methicillin was not orally active. Ampicillin was not only quite effective given orally but had a much broader spectrum and led to a series of yet more improved agents.

Clearly from this it was apparent that semisynthesis in the hands of organic and medicinal chemists could usefully build upon the structures available from nature and thus produce superior agents. Thus ultimately tens of thousands of such agents were produced in collaboration between fermentation biochemists and workers in synthesis.

Total chemical synthesis, on the other hand, was of lesser utility. All of the important antibiotics were synthesized one after the other, but their structures were so complex and the needed chemistry sufficiently lengthy and involved that no practical use came of this (except in the special case of chloramphenicol) for many years. Notable advances in synthetic technology resulted, but clinical effectiveness was little influenced by this work.

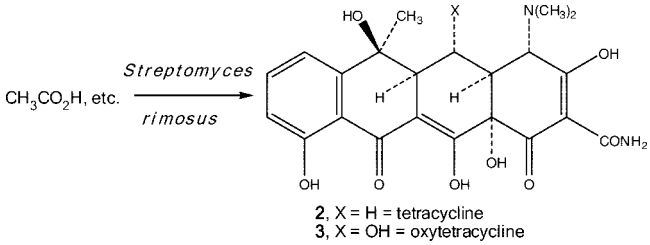
Directed Biosynthesis

Partial chemical synthesis of the tetracyclines at that time was particularly challenging due to the chemical instability of these molecules to ordinary reagents, their difficult solubility, and lack of easy means of separating pure products from complex mixtures. An apparent missing link among the tetracyclines available from fermentation was oxychlortetracycline. It had long been sought through screening but without success. When the biosynthetic pathway to the tetracyclines became known in general detail,[27] a logical reason for the fact that it appeared not to be produced naturally became apparent. Tetracycline (**1**) and chlortetracycline (**2**) were normal fermentation products of *Streptomyces aureofaciens*, and 6-demethylchlortetracycline and 6-demethyltetracycline were produced by laboratory-generated mutants. Thus, *S. aureofaciens* had the capacity to chlorinate but apparently not hydroxylate at C-5. Oxytetracycline (**3**) and tetracycline were normal fermentation products of *Streptomyces rimosus.* This organism could hydroxylate but did not chlorinate.



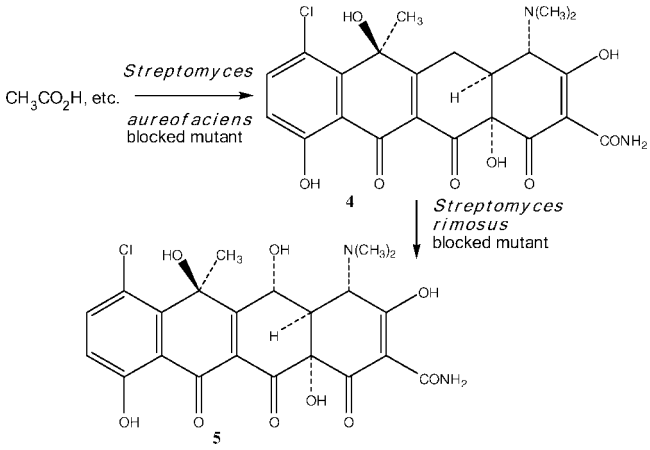
1, X = Cl = chlortetracycline
2, X = H = tetracycline

We reasoned that a *S. aureofaciens* mutant blocked past the chlorination step (which was known to be relatively early) might produce an intermediate stable enough to be isolated that was not carried on to the final products in these cultures. If fed to a blocked



2, X = H = tetracycline
3, X = OH = oxytetracycline

mutant of *S. rimosus* in which the hydroxylation enzyme was still operative, this intermediate (**4**) might be carried on to the desired oxychlortetracycline (**5**). Screening for success was rendered plausible since neither culture produced antibiotic activity by itself, so strains that produced activity when fed the appropriate precursors were likely to contain the desired compound. After considerable labor, this was found to be the case.[28–30]



Isolation of the active product was exceptionally difficult because it was not stable at pH levels where it separated from contaminants by column partition chromatography. Ultimately this was solved by dripping the column eluants into buffer solutions to adjust the pH to a safe range rapidly. When separated from these buffer solutions, the product could be crystallized and was shown to possess the long sought after structure.

Unfortunately, instead of combining the most favorable properties of its parents, oxytetracycline and chlortetracycline, it proved to be too unstable for oral administration to patients despite its attractive antimicrobial properties. Thus this effort at directed biosynthesis represented a scientific success but a commercial failure.

This attempt to produce unnatural natural products today would be done using gene splicing techniques. Producing novel macrolides in this manner is now readily done by those possessing sophisticated genetic information lacking to us in those early days.[31–33] One can speculate that at some prehistoric date, streptomyces had the capacity to produce 5-oxychlortetracycline but found this nonproductive for the same reasons that we had discovered. Could it be that nature had performed such experiments long ago and abandoned this for the production of more useful products? We will never know but should concern ourselves in project planning today to consider whether the targeted structure would indeed possess useful properties.

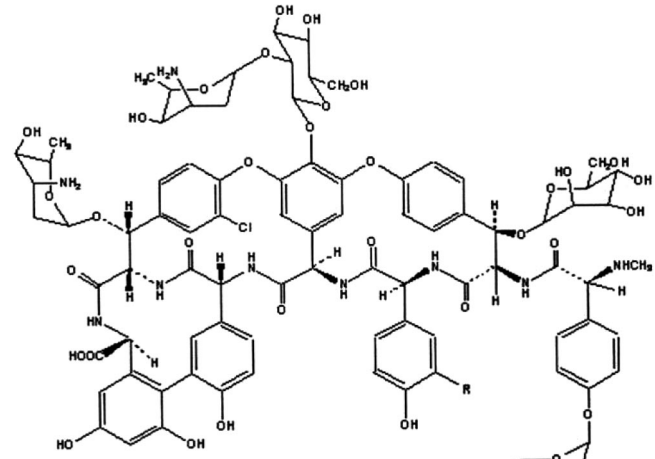
The power of semisynthesis is clearly demonstrated in the tetracycline field with the introduction and prospering of doxycycline, minocycline, and tigecycline into clinical use.[34]

Isolation of Novel Agents

The classical stratagem for finding antibiotics with extended spectra, including resistant organisms, was the screening for and evaluation of novel agents. The underlying belief was that molecules possessing novel structures would possess different activities and, perhaps, even novel modes of action. There is significant truth in this, but it does not always follow.

The hope that nature has already discovered "utopiamycin" so that it is out there for us to find commissioned a great many drug-seeking campaigns, in some of which the author participated.

A significant early campaign produced avoparcin, a glycopeptide antibiotic[35–38] that is related to the better known vancomycin. The genesis of this program and its consequences are instructive. An early use of tetracycline-containing residues was their administration to farm animals. This was shown to result in growth stimulation such that relatively low (subtherapeutic) doses in their feed led to accelerated growth to marketable size at an affordable cost.[39] Relatively early it was found that tetracycline-resistant organisms were sometimes harbored by these animals. Concerns that this might lead to drug-resistant human infections caused us to search for alternative agents. This was prudent since some time later such use of tetracyclines was banned in the United Kingdom and other countries although tetracyclines continue to be so used in the United States.[40]



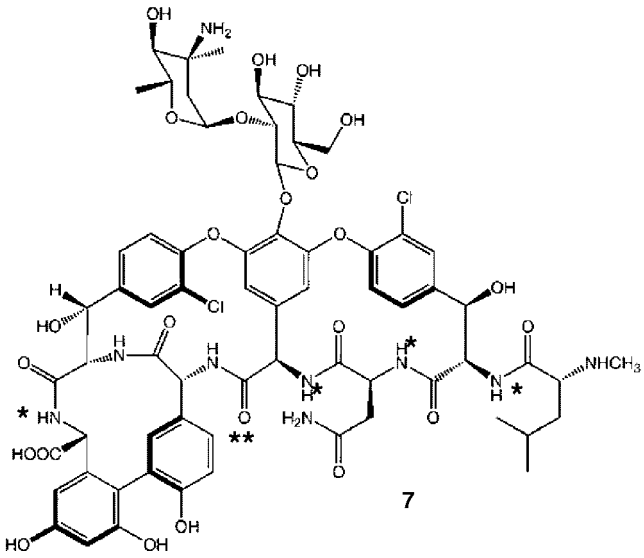
6α, R=H; **6β**, R=Cl

Avoparcin (**6α**, **6β**)[35–37,40] was an attractive alternative and found extensive veterinary use in Europe. At this early date vancomycin was still a relatively minor drug since the severe therapeutic problem represented by methicillin-resistant *Staphylococcus aureus* (MRSA) developed much later. Avoparcin was not absorbed if taken orally and was conveniently soluble for incorporation in formulations, and, more importantly, no significant resistance development was associated with this antibiotic class. Demonstration that it was effective in weight stimulation was therefore welcome.

It has taken decades for resistance development to vancomycin−avoparcin to appear. Resistance was noted to be significant first in Denmark, and its incidence was apparently traced to the use of avoparcin.[42] At first only found in enteric bacteria, ultimately the genes for resistance surfaced in *Streptococci* and then in *Straphylococci.* The latter posed a severe threat to humanity because by then resistant staph had become a significant pathogen and vancomycin-class antibiotics were among mankind's last line of defense against MRSA.

The development of avoparcin resistance demonstrates that discovery of novel structures can, for a time, deal with bacterial resistance to chemotherapy but that ultimately resistance to these new agents will also appear. It seems that the rate of such resistance emergence is directly proportional to the extent of use of a particular antimicrobial agent. This requires more conservative prescription of antibiotics than is the present practice.

The underlying molecular biology of vancomycin−avoparcin resistance is instructive. The binding of vancomycin (**7**), and presumably avoparcin, to its molecular target [acylated D-alanyl-



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D-alanyl peptides (**8**), which are essential intermediates from which bacteria construct their cell walls] is known. A very late step in normal cell wall construction is cleavage of the D-ala-D-ala bond attached to one strand and exchange of the last D-alanyl residue with a glycine unit from an adjacent cell wall strand. In this way the wall goes from a two- to a three-dimensional construction with accordingly much greater strength. The interaction of vancomycin with this acylated dipeptide involves hydrogen bonds to four of the amide NH groups and one of the amide carbonyls of vancomycin. These are indicated by single or double stars (*) in formula **7**. This drug−receptor complex prevents cell wall formation.

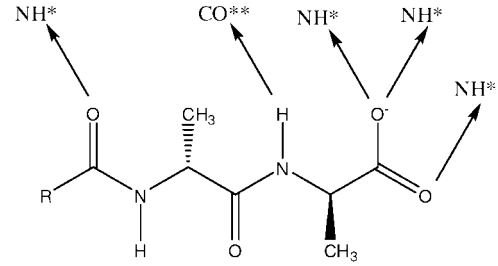
Eventually bacteria have been able to substitute a D-lactyl residue (**9**) for the penultimate D-alanyl moiety of the normal cell wall precursor. The consequence of this is that a key hydrogen -bonding interaction [indicated by a double star (**) in formula **7**] is lost. As a consequence, binding affinity is 1000-fold decreased and resistance occurs. Avoparcin (**6**) has hydrogen-bonding sites analogous to those of **7**, rationalizing the development of cross resistance. This kind of resistance was slow to emerge because the microorganisms had to alter the fundamental biochemistry of their cell wall formation in order to get around the toxicity of avoparcin. They also had to learn how to share this trait with other pathogens. Nonetheless they ultimately succeeded in doing so.[43]

This extremely slow case suggests that discovery of new antibiotics only buys time. Eventually if not soon, resistance will develop. This is usually proportional to the degree of usage of the new agent. Thus only fairly obscure antibiotics will retain their potency for a significant period.

In this context it is important to observe that almost half of all the antibiotics produced in the United States are presently administered to animals in the form of feed supplements for growth promotion. The wisdom of continuing to do this should be reconsidered from the perspective of the cost−benefit ratio involved.

Much later, in conjunction with Squibb scientists, we had the pleasure in participating in working out the structures of the dactylocyclines (**10** is dactylocycline A).[44] These are the only known tetracycline glycosides and have several other unusual structural features that are, however, consistent with biosynthetic knowledge. These agents have attractive antimicrobial spectra but are too unstable in acid solutions to survive oral administration. Thus these interesting molecules also failed to see clinical use.

In another adventure in the tetracycline area, Abbott Laboratories workers had isolated chelocardin (**11**) but could not come up with a



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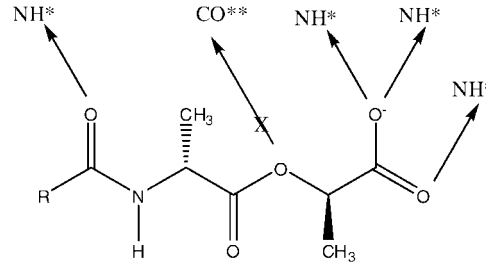

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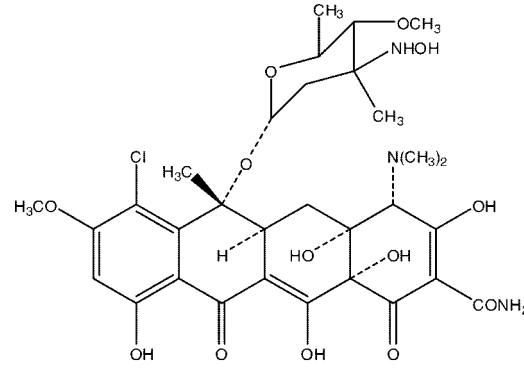

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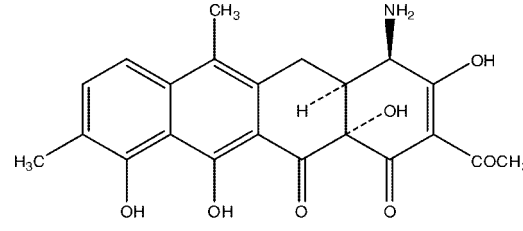

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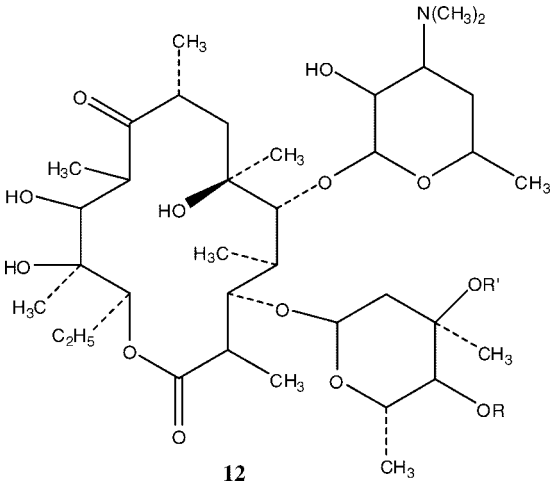
convincing structure for it in reasonable time. With our assistance it was shown to be an anhydrotetracycline derivative with an unusual β-primary amino group and some other unusual structural features that could also be rationalized by biosynthetic knowledge.[45–47] The utility of exciton coupling circular dichroism measurements in these experiments is particularly notable. Chelocardin advanced into phase II clinical trials before being abandoned due to insufficient superiority over existing agents, poor solubility, and a short remaining patent lifetime.[48] Subsequent work led to semisynthetic analogues with enhanced in vitro activity, so this story is not necessarily finished.[49]

There are surely other natural tetracyclines out there that have interesting structures and are yet to be discovered if one can devise suitable means of screening for them.

The search for novel antibiotics from natural sources initially concentrated on fungi and streptomycetes. The intensity of this effort soon led to diminishing returns, and the introduction of truly novel agents (not related to already known structural classes) fell off considerably in the 1970s.



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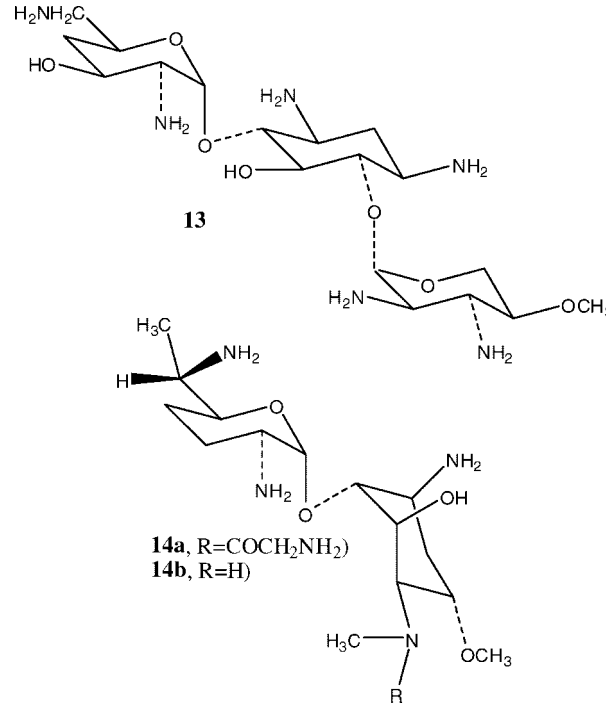
An approach to dealing with this problem then was to search for increasingly rare microorganisms inhabiting increasingly rare habitats. The reader will recall that chelocardin is a product of a micromonospora, and dactylocycline is produced by a *Dactylosporangium* sp., as relevant examples. Among the fruits of the Abbott-Kyowa effort were the discovery of some additional megalomicins (**12**, in which R and R^1 are selected from hydrogen, acetyl, and propionyl ester groups) from a Micromonospora,[50,51] seldomycins (**13** is factor 5)[52–55] and the fortimicins (**14a** is fortimicin A and **14b** is fortimicin B), which are used clinically in Japan for the treatment of Gram-negative infections,[56–59] also from a Micromonospora.[60–64]

Chemical ionization mass spectrometry, novel at the time, was very helpful in elucidating the magalomicin's structures, and circular dichroism spectra of molecular complexes formed with copper(II) ion in cupra a solutions contributed strongly to the structure determination and absolute stereochemistry of the seldomycins and the fortimicins.

The take-home lesson from these productive collaborations is that useful new structures are indeed present in such unusual biota but that they are mostly variants on already known themes. Nonetheless medicinally useful agents can be found in this way.

Semisynthesis

As noted previously, semisynthetic modification of existing antibiotics can be very beneficial in improving their properties. A case in point is erythromycin and related macrolides. Semisynthetic improvement can be done either adventitiously (empirically as allowed by synthetic accessibility) or, more interestingly, by following a definite hypothesis. The products of certain reactions and properties of erythromycin were initially mysterious, hindering development of a predictive understanding of their chemistry.[65] This was resolved in large measure when the solution conformation became known. Initial brilliant work using NMR techniques by Richard Egan and Thomas Perun and their colleagues of Abbott Laboratories clarified much of this but did not lead to an unambiguous solution. Clearly the molecule could not possess a simple variant of the putative diamond lattice (**15a**), as the bond angles were not compatible with this. A diamond lattice would provide for the most compact and energy minimal ring conformation; however, even placing the lactone moiety so that there would be few conformational clashes between inward directed substituents (**15b**), there would remain significant 1,3-diaxial interactions, for example, between methyl-4 and methyl-6. The bond angles of erythromycin were found to be largely temperature invariant up to 100 degrees, so the molecule did possess a stable conformation.[66] Working out just what this might be using the chemical shifts and the Karplus equation relating coupling constants to bond angles was insufficient for interesting reasons. The Karplus equation gives two possible angles for many coupling constants. In small ring



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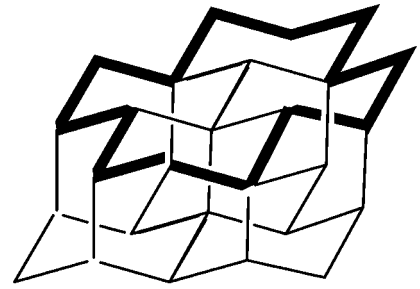
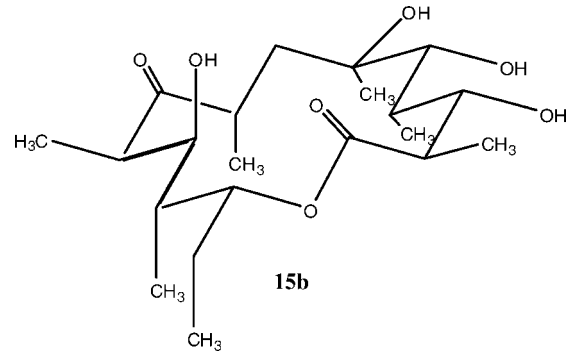
14a, R=$COCH_2NH_2$)
14b, R=H)



15a



15b

compounds one of the answers is impossible so no ambiguity results. In 14-membered macrolides, however, both solutions are theoretically possible, with the only limitation being that the ring had eventually to come together. Circular dichroism measurements resolved this difficulty and produced a unique conformational solution.[67,68] Both the ketone and the lactone chromophores are strongly influenced by the arrangement of nearby atoms in space as dictated by the octant and lactone sector rules. Since the two chromophores absorb at substantially different wavelengths, the resulting peaks do not overlap, so the spectra are easily factored. Furthermore, they are widely separated in the lactone ring, so each reveals nearby structural features in a different portion of the molecule. Examination of the circular dichroism spectrum of a variety of macrolide analogues provided the missing information

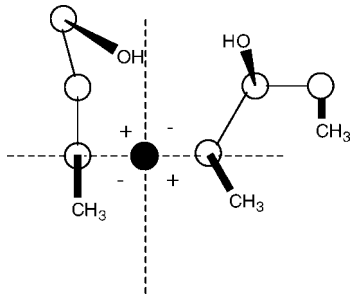
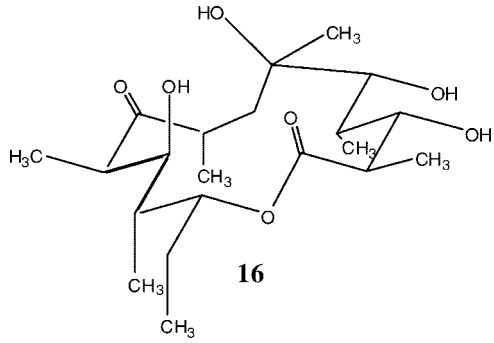


Figure 4. Octant presentation of the vicinity of the carbonyl moiety of erythromycin.

that led to a unique conformational solution. The octant projection of the keto vicinity is presented in Figure 4.



16

The deviation from the diamond lattice that this revealed relieves the 1–3 interaction between the methyl groups at C-4 and -6 by a modest outward rotation of carbon 6 (**16**). The presence of only hydrogens at C-7 facilitates this because it does not introduce intolerable new interactions when C-7 is rotated. Thus nature has been clever in leaving this carbon unsubstituted to allow for this.

In addition to allowing a ready rationalization for the outcome of certain otherwise puzzling reactions, this provided a ready rationale for the clinical observation that erythromycin rapidly forms an irreversible cyclic ketal in acid as in, for example, the stomach. This ketal formation leads to inactivation, and the resulting spiroketal causes stomach cramps. The newly discerned conformation shows that the C-6 hydroxyl substituent lies above and very near the C-9 ketone moiety. This is not apparent when looking at a flat depiction of the molecule. The proximity of these two centers readily rationalizes the rapid ketal formation in acidic media following protonation of the carbonyl. This change can be detected quite sensitively in circular dichroism measurements as the keto chromophore is lost in this process. Thus, circular dichroism measurements allow for a convenient analysis of the rate and extent of this clinically relevant reaction.

A useful application based in part on this knowledge was the selective methylation of the C-6 hydroxyl group, leading to clarithromycin.[69,70] This prevents the cyclic ketal formation and leads to an acid-stable drug that has seen very significant clinical use. Later a different group of investigators took a different approach. Rearrangement of erythromycin so as to replace the keto moiety with an amino function produces acid-stable azithromycin.[71] The very favorable pharmacokinetic character of azithromycin has led to its dominating the macrolide market.

Antimicrobial Agents from Higher Plants

In parallel with uncovering novel antimicrobial agents from novel sources, we turned to an investigation of a fruitful source of antibiotics from even less studied sources, the higher plants.[72] These agents are by and large quite different from those produced by soil microorganisms. Surely some of these will have pharmacokinetic properties that would allow them to be useful for human infections, particularly with the aid of semisynthesis to enhance their natural characteristics. Some of the most potentially useful of these agents are detailed below.

Strobilanthes cusia (Acanthaceae) has seen traditional use in Taiwan for a variety of infections. In our screening program it was shown to have activity against *Mycobacterium smegmatis,* a screening stand-in for potential antitubercular activity.[73,74] Our interest in the active constituent, the alkaloid tryptanthrin (**17**), was heightened when Scott Franzblau demonstrated that it was 4 times more active in vitro against *M. tuberculosis.* Tryptanthrin was an old compound and not readily patentable, so we undertook a synthetic program partially in collaboration with William Baker and his colleagues at the PathoGenesis Corporation[73] and finally using combinatorial methods in our laboratory[75,76] The existing syntheses were cumbersome, so efficient new convergent routes were developed. The best compound emerging from this effort, PA505 (**18**), was 10-fold more potent than tryptanthrin itself and gave better blood levels orally in mice as well as being rather nontoxic.

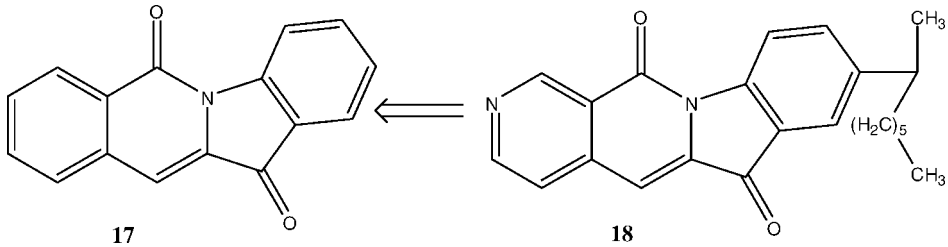
Although effective in infected murine disease models, it was bacteriostatic in vivo and was judged not to be worth further clinical development. This was particularly felt because treatment of tuberculosis in AIDS patients probably requires the use of bactericidal agents. This outcome was disappointing but did confirm our belief that novel agents in higher plants had significant potential for use in treating infected humans.

A wide variety of antimicrobial agents with diverse structures was obtained from several higher plants, but most of these did not show enough potential for development. In this they paralleled many of the agents isolated from fermentation of soil microorganisms.

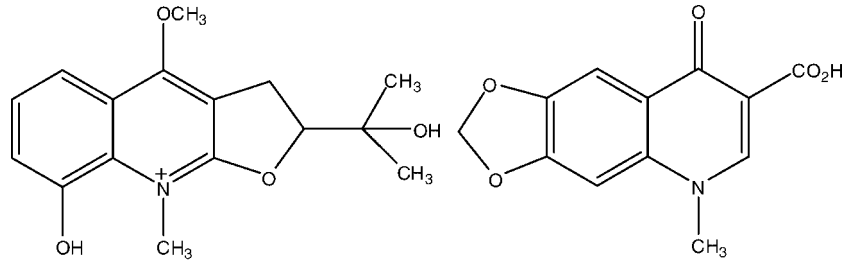
Of the various agents explored, the one that ultimately opened up the most productive series of experiments, although along wholly unexpected lines, was pteleatinium chloride (**19**) from *Ptelea trifoliata*, the hop tree.[77] This tree had found significant use in the years following demobilization of Hessian troops at the conclusion of the American Revolutionary War. They had been given homestead land in the newly acquired western territories that included modern Ohio. Among their desires was the ability to brew beer. This, of course, required a source of hops that were locally unavailable. The hop tree served as a substitute until communication routes were developed that made real hops reasonably available. Today the name of the tree remains in use, but farmers are generally unaware of how it got to be called this.

In addition to its flavor, hops (*Humulus lupulis* L.) is mildly antiseptic, helping to keep the brew from bacterial spoilage during the brewing process. Thus, we were interested to see what level of activity might be present. Although there had been several previous chemical studies of the contents of *P. trifoliata*, we found the main alkaloid, pteleatinium chloride, to be novel and abundant and to possess the activity. It was not particularly potent, but there was a lot of it. Obviously when screening, an interesting level of activity in crude extracts can be due to a small amount of an intensely active substance or due to a large amount (or number of) modestly active substances. In this instance, the latter was the case.

While working out the structure of this substance, new synthetic chemistry was developed in our laboratory. It was noticed that the structure was vaguely similar to that of oxolinic acid (**20**), used at that time clinically as an orally active urinary tract disinfectant. The published synthesis resulted in a single position for the methylenedioxy moiety on the aromatic ring, whereas our chemistry would allow us to move this substituent to the other two positions and see what effect this would have on potency. In the event, it was found that the position in oxolinic acid was the best one.[78]

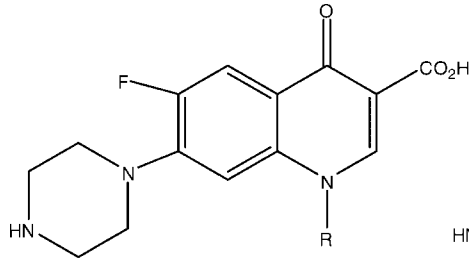


17 **18**

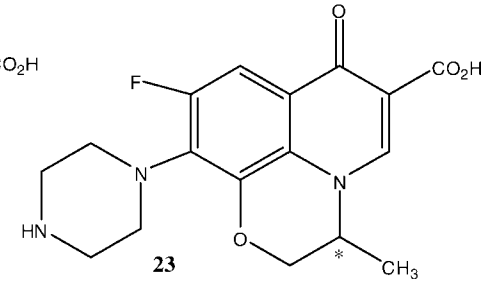


19 **20**

At the time this work was performed, there was relatively little general interest in the quinolone anti-infectives. Shortly thereafter, however, the exciting antimicrobial properties of norfloxacin (**21**) and subsequently ciprofloxacin (**22**) and then ofloxacin/levofloxacin (**23**) dramatically increased interest in this class, and soon a significant percentage of the world's industrial medicinal chemists were engaged in making analogues.[79]



21, R = Et
22, R = cPr



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We were drawn to the structure of ofloxacin since it had a prochiral center (*). Its mode of action was becoming known following the discovery of DNA gyrase and its essential role in the housekeeping biochemistry of bacterial DNA. Since it had an enzymatic target, it seemed reasonable to suppose that chirality might play a significant role in its action. Chemistry, adapted from that of H. Grohe of Beyer-Germany,[80] developed in conjunction with Daniel Chu of Abbott Laboratories enabled us to prepare both isomers efficiently starting with natural and epimeric alanine.[81] This settled the most potent and least toxic absolute stereochemistry as *S* and resulted in a very efficient synthesis for this agent (**24**–**27**), which has become very important clinically under the name levofloxacin.

A side effort subsequently led to combinatorial methods for synthesis of analogues in this class as well as the development of an inexpensive and convenient apparatus for such studies.[75]

Unfortunately a number of pharmaceutical firms have abandoned natural product work in favor of investments in combinatorial chemistry. Clearly combinatorial chemistry is an efficient and cost-effective means of making numerous potential hits for screening and analogues of lead substances for the examination of structure–activity relationships; however its principal virtue is speed of molecular construction. This is useful, but it does not intrinsically produce diverse chemical structures of potential biological utility. The initial promise that combinatorial chemistry would be more efficient in producing novel and marketable antimicrobial agents as compared to natural product screening has not been realized. It is apparent that a number of firms are now rethinking this proposition, but it is very difficult to reconstitute teams with the necessary specialized skills needed to carry out natural product screening efficiently on an industrial scale.

Our work and that of others has demonstrated that instead of replacing natural product studies by combinatorial chemistry programs, the combination of these methods is powerfully useful in rapidly exploring SAR and PK phenomena.

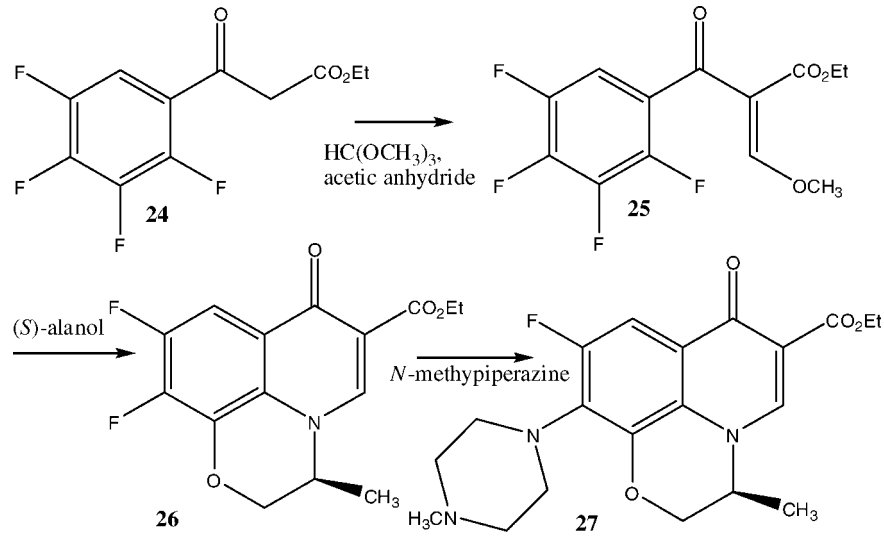
A number of explorations of the quinolone ring system have been undertaken in our laboratory using this technology. This, however, drifts away from the main theme of this review, so will only be referenced here.[82,83]

A detailed knowledge of the molecular mode of action of important drugs often gives precious insights into the relationship between structure and function, thus serving as a guide to analoging. Consequently we turned our attention to this direction in studying the fluoroquinolones.

Linus Shen and his colleagues at Abbott Laboratories, following detailed biochemical studies of these agents, concluded that they most likely cooperatively self-associate into at least a dimer and possibly a tetramer when DNA gyrase was operating on DNA.[84–89] This operation resulted in the transient preparation of a single-stranded segment (as a "bubble" or perhaps as a "gate") to which the quinolones bind tightly.

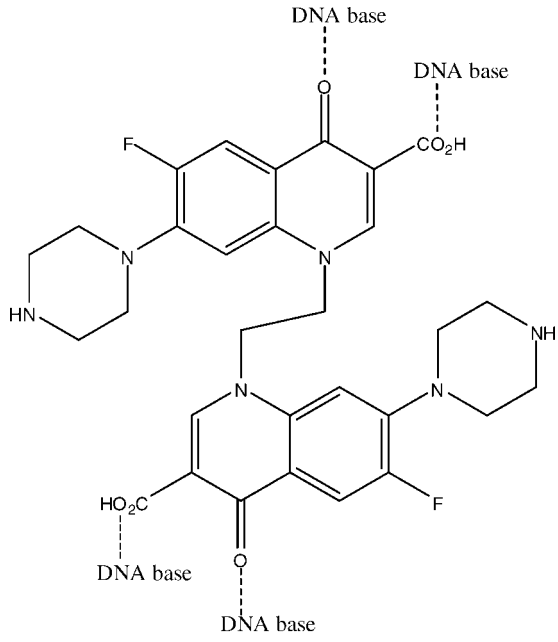
Binding in this manner (illustrated in Figure 5) is believed to freeze the assembly into a ternary complex that cannot revert to the components nor progress to viable final products. Denying access of bacteria to their DNA presents great difficulties for bacteria and triggers rapid cell death by mechanisms that are as yet imperfectly understood.

Examination of the X-ray structure of nalidixic acid, the first of the clinically active quinolones, showed that the unit cell was populated by four molecules self-associated along their lipophilic



24 25 26 27

lower periphery in a head-to-tail manner. By computer graphics it could be shown that the *N*-ethyl substituents could be joined covalently at their methyl groups with only a minor displacement of the other atoms of the molecules.



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This was readily accomplished in the laboratory by synthesis but patterned after the more potent norfloxacin instead, and a series of homologues were made in which the linking methylenes ranged from 3 to 5. Gratifyingly, when tested in vitro against DNA gyrase activity, the synthetic dimer with four methylenes was the most active against the enzyme isolated from Gram-negative *Escherichia coli,* just as theory had predicted. Interestingly, when DNA gyrase, prepared from Gram-positive *M. luteus*, was examined, the most active dimer was linked with five methylenes instead. It was discovered that another enzyme was often involved with inhibition of Gram-positive bacteria. Bacterial topoisomerase IV was not known at the time of these experiments. Put together, these findings possibly rationalize the observation that it has not been possible as yet to prepare quinolones with equivalent potency against both Gram positives and Gram negatives. These host–guest relationships are clearly complex and are yet to be worked out in sufficient detail.

The general but not universal acceptance of the self-assembly model for quinolone action serves to rationalize conveniently and perhaps truthfully the role of chirality at the N-1 position, as this would dictate the chirality of the self-assembly stack so as to allow for closest approach of the two partners to one another.[90]

In time, the extensive exploration of the chemistry and biology of the fluoroquinolones inevitably led to great difficulties in producing significantly new agents with attractive properties. The newest agents possess relatively modest increases in spectrum and all too often significant toxicity. Trovafloxacin and temafloxacin are examples where severe toxicity led either to withdrawal or to significant restriction of use following clinical release. This prompted a search for analogues with rather different structures.

The Shen model described above rationalized the finding that substituents at a C-2 carbon led to dramatically reduced activity. Such substituents would interfere with the coplanarity of the C-3 carboxyl group with the ring system, thus hindering hydrogen bonding to the DNA portion of the terniary complex. Reasoning that this could be avoided by constraining such a substituent into a ring, exploration of bioisosteric carboxyl surrogates in the form of heterocyclic rings was undertaken.

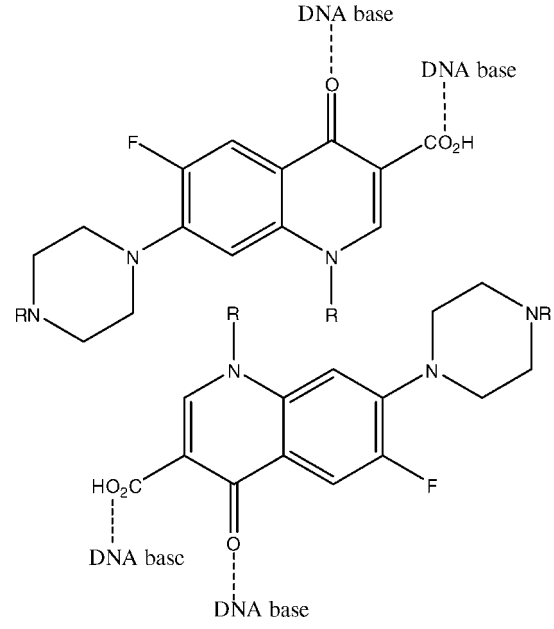
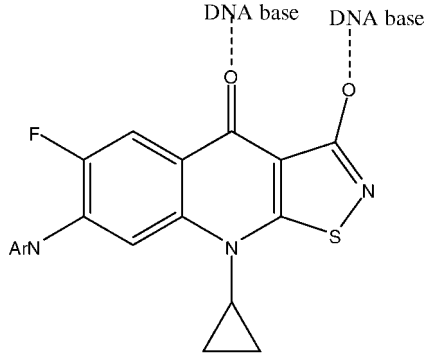


Figure 5. Self-association of a pair of generic quinolone molecules each hydrogen bonding to a single-stranded region of DNA such that the melt region cannot progress or regress to its original duplex form.

Synthesis of isothiazolone rings attached to C-2 and C-3 (**29**) required development of novel chemistry but was rewarded with the finding that the products were very highly bioactive.[91] There was, however, significant cytotoxicity. The Abbott group pursued these as potential anticancer agents, while another persisted with antimicrobial studies. The latter resulted in molecules with very significant activity in vitro against MRSA.[92–94] Neither effort has yet resulted in clinically useful agents.



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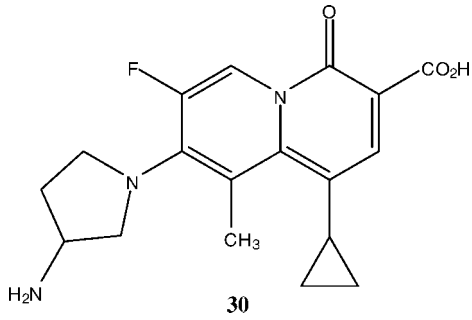
Another relatively simple artifice was to move the quinolone nitrogen from the 1 position to the C-4a position. This bioisosteric exchange would not significantly alter the bond angles and the atomic positions but would potentially strengthen the ability of the products to hydrogen bond to DNA. Making these molecules also required the development of novel chemistry leading to a very substantial improvement in antimicrobial potency that included otherwise fluoroquinolone-resistant organisms. The best of these agents, ABT-719 (**30**), has been evaluated in the clinic but has not been marketed, apparently due to unattractive side-effects.[95]



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Additional explorations included different annealing of the benzene ring, its attachment by a single C−C bond, and the like. While generating interesting and novel chemistry, this did not lead to clinical candidates.[82]

Much remains to be done in the quinolone field.

In sum, many avenues have been explored in attempts to cure infectious diseases. These have been significantly thwarted by the emergence of drug-resistant strains. None of the approaches previously used either individually or collectively have proven fully effective. They have, however, dramatically reduced mortality from these causes in the final two-thirds of the 20th century.

Attacking resistance mechanisms in effect is attacking a symptom rather than a cause. Perhaps use of these agents could be profitably enhanced by supplementation with the approaches in the next two sections.

Antimutagenesis

It has long been believed that resistance genes have been present in bacteria before the human use of antibiotics based upon the presence of these compounds in soil microorganisms. As a consequence, the use of antibiotics would in time select in favor

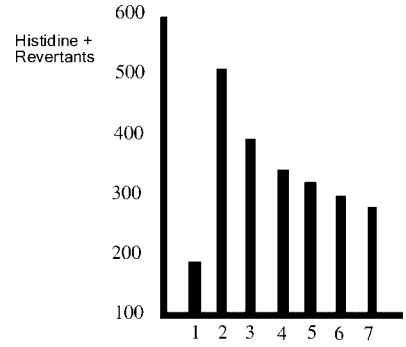


Figure 6. Revertant mutations of *Salmonella typhimurium* TA 102 strain induced by hydrogen peroxide and prevented by increasing concentrations of epigallocatechin gallate. Lane 1 = cells only, lane 2 = cells plus hydrogen peroxide, lane 3 = 2 + **31** (2.5 μM), lane 4 = 2 + **31** (5 μM), lane 5 = 2 + **31** (10 μ), lane 6 = 2 + **31** (20 μM), and lane 7 = 2 + **31** (40 μM).

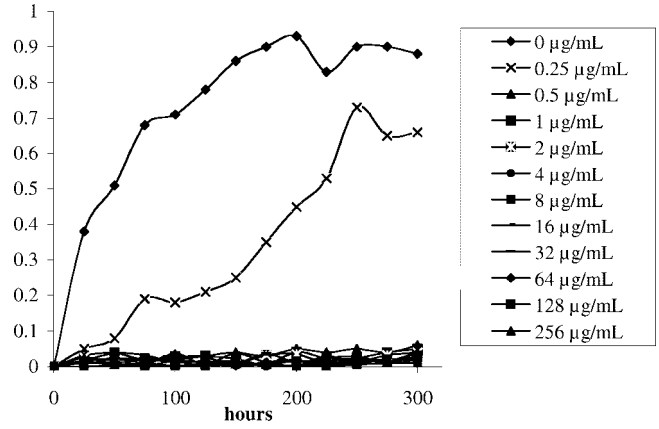


Figure 7. Inhibition of resistance development of *Staphylococcus aureus* to doxycycline by graded concentrations of epigallocatechin gallate.

of these intrinsically resistant cells. This idea is logical and may be true. It may, however, not be the whole story. It is hard to accept the idea that resistance genes were developed by bacteria in ancient times against modern synthetic agents such as the sulfonamides, linazolid, metronidazole, and the fluoroquinolones. It is more credible to believe that random mutations are occurring all the time, and those are preserved upon exposure to a noxious substance when they prove to be protective. Both processes may be occurring at the same time.

Should this be true, it is likely that some of the naturally occurring antimutagenic agents prevalent in plants[96] could minimize or even prevent such mutations to resistance. Unsurprisingly, hydrogen peroxide leads to mutations in *Salmonella typhimurium* TA 102. These concentrations also produce strand breaks in DNA in vitro. Faulty repair is a plausible explanation for these phenomena. Addition of increasing quantities of epigallocatechin gallate (EGCG) (**31**) exerts a progressive antimutagenic action and also prevents DNA strand breaks at otherwise no effect concentrations (Figure 6).[97] Other catechins produce similar results but at weaker concentrations. These experiments suggest that this would be a useful area to explore in dealing with resistance in vivo.[19,97] Coadministration of antimutagenic agents already in the food chain and shown to be safe for human consumption (catechins are rich in green tea) with antibacterial agents would be a harmless and potentially useful means of dealing with the resistance phenomenon.

Reference back to Figure 2 recalls that undertreatment with doxycycline leads ultimately to emergence of resistant strains of *Staphylococcus aureus.* Figure 7 demonstrates that this effect is

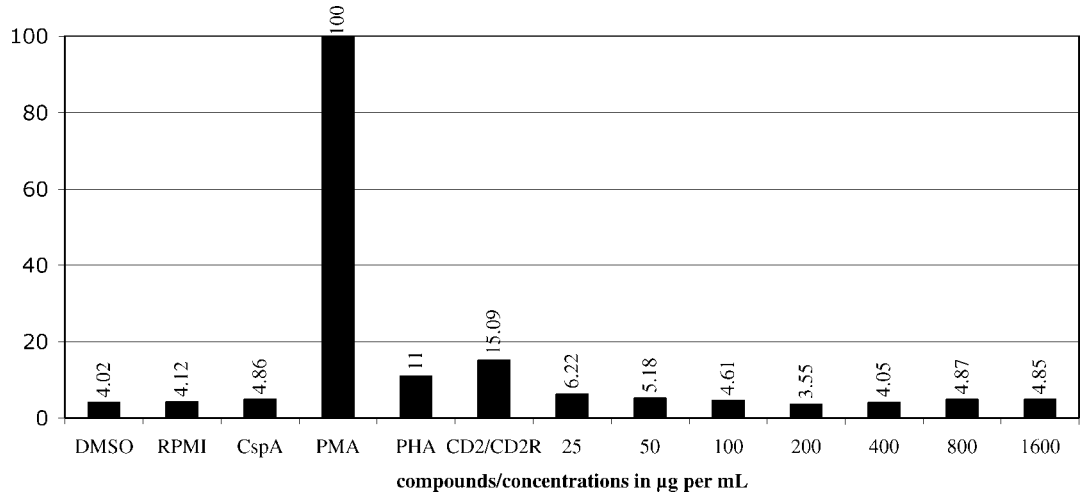
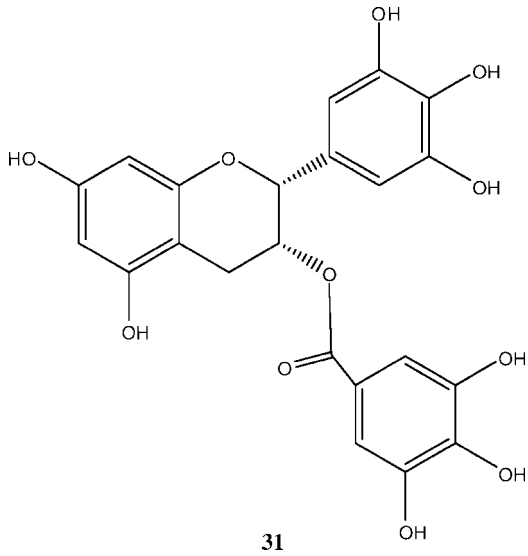


Figure 8. Concentration dependence of immunostimulatory power of solvent extract of *Echinacea purpurea* stem and leaf powder against human $CD4^+$ cells ex vivo. DMSO = dimethyl sulfoxide, RPMI = Roswell Park Memorial Institute media blank, CspA = cyclosporine A, PMA = phorbol myristyl acetate, PHA = phytohemeagglutinin, CD2/CD2R = cell surface antibodies available from Becton Dickinson Corp.

dramatically inhibited by administration of 16 μg/mL of the antimutagenic compound EGCG (**31**).



31

Immunostimulants

Enhancing the body's immune system to help ward off or attack bacterial and viral infections would also seem to be logical. Prevention of disease is usually more fruitful than attempting to cure disease once it has taken place.

A popular herbal remedy consumed in very significant quantities by lay persons is Echinacea (*Echinacea purpurea* Moench.). Although the literature is certainly not unanimous on this, there is much support for the proposition that prophylactic use of echinacea in advance of the cold season shortens colds and makes colds less severe due to the presence of immunostimulating constituents.

One of the problems bedeviling convincing clinical validation of this use is the fact, common among herbal remedies, that the commercial preparations used are often not analyzed, and when they are, the analyte is often an indicator component with no assurance that its quantity is therapeutically meaningful. This seems to be the case with echinacea.[98]When analysis is done, it is usually performed by HPLC examination of the solvent-soluble components in part because they possess a chromophore that is easy to quantitate. There is a significant literature indicating that the plants also contain substantial amounts of water-soluble glycans that possess immunostimulant potency. These agents have been shown

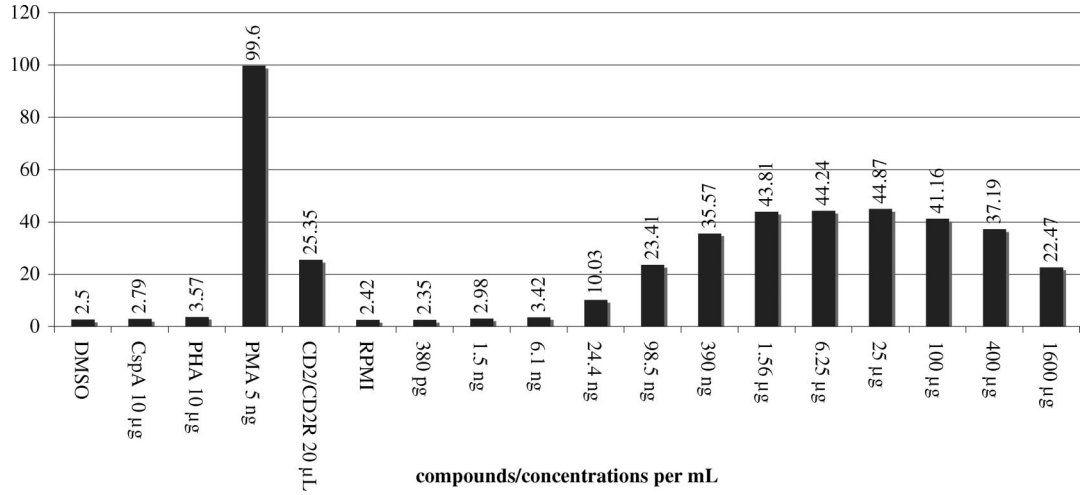


Figure 9. Concentration dependence of immunostimulatory power of water extract of *Echinacea purpurea* root powder against human $CD4^+$ cells ex vivo. The acronyms are explained under Figure 8.

by flow cytometry to be more potent that the solvent-soluble components.[99] In Figure 8, it can be seen that an ethyl acetate extract of the above ground parts of *Echinacea purpurea* possesses definite but rather weak immunostimulatory power, and this at rather high concentrations. By contrast, in Figure 9 it can be seen that the aqueous extract is powerfully stimulatory under the same conditions. Echinacea can be analyzed by engulfment of carbon particles ex vivo, by HPLC using evaporative light scattering detection, or by immune cell differentiation using flow cytometry. This is rarely done commercially. Using relevant bioassays in place of spectroscopic measurements is more likely to be useful.

It is interesting to speculate that synthesis of antibiotics that also possess immunostimulatory and/or antimutagenic activity as well might provide a novel and useful approach to the resistance problem.

Summary and Conclusions

Intensive work spanning two-thirds of a century in combating infectious diseases by development of antibiotics leads to the conclusion that microbial resistance is inevitable unless some unforeseen discovery lies ahead. This is a war that will never cease. Bacteria will not eradicate mankind and mankind will not eradicate bacteria. The introduction of new antimicrobial agents will continue to help keep morbidity and mortality as low as possible, but resistance to these new agents will inevitably develop.

Continued close observation and exploitation of natural phenomena appear at present to be the wisest course for scientists to follow in trying to deal with this problem.

Acknowledgment. In presenting these experimental findings the author is very conscious that they were group efforts involving many brilliant co-workers. He is also mindful of the danger of overclaiming credit as expressed forcefully by von Clausewicz. "Victory has many fathers but defeat is an orphan!" The author has been blessed with a long life, challenging work, the opportunity to know and work with brilliant students and collaborators, to have had parents and family who have endured my preoccupations and moods and who have been unfailingly supportive. Most particularly, I thank my longest and best collaborator without whose assistance none of this work would have been possible—Betty J. Mitscher.

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