

## Chapter 2

# Rational Approaches to Antibacterial Discovery: Pre-Genomic Directed and Phenotypic Screening

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### 2.1 Introduction

Early antibacterial discovery might be divided into the synthetic and antibiotic traditions. The salvarsan-prontosil strand led to exploration of antimetabolite and other chemotherapeutic approaches, while penicillin's discovery led to the fruitful exploitation of natural products produced by microorganisms. With the investigation of the mechanisms of action of synthetic and antibiotic agents and the principles of selective toxicity and specificity of action, the separation becomes less clear. Both modes of discovery yield small molecule inhibitors of essential bacterial functions; both started with empirical discoveries but eventually the search evolved to favor more directed methods of compound selection and design. Over time, the yield of novel antibacterial classes via both synthetic and natural product routes has declined, paralleling the increase in more rational screening methods. Is this a causal relationship or merely a correlation?

The history of these directed attempts, especially in the natural products area of antibacterial antibiotic discovery, has been obfuscated due to the conscious effort by pharmaceutical companies to hide their methodology from competitors. Although commercially relevant discoveries were revealed through patent and publication with reasonable speed, it is often only through retrospective reviews written much later by the discoverers – or their informants – that the methods used were uncovered. Often, we do not know when such screens were first used. Luckily, much of the screening methodology used to detect inhibitors of peptidoglycan synthesis has been published over time and forms the bulk of the material in this chapter. In the 1980s through 1990s, when there was actually a good deal of innovative screening

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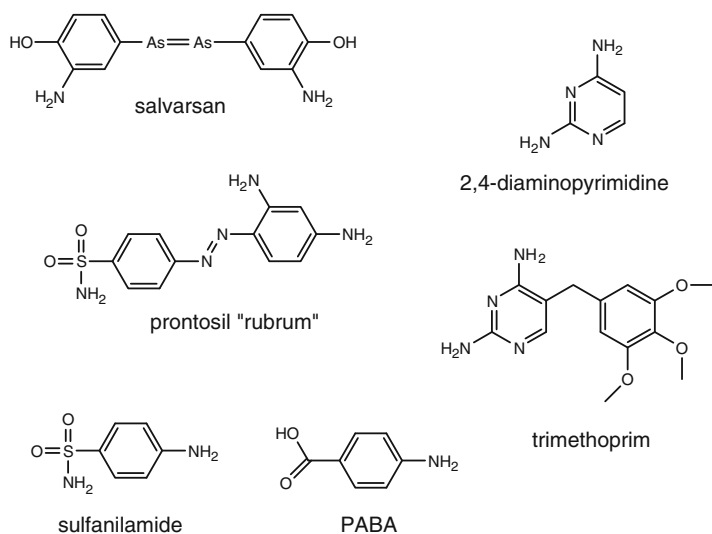
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being carried out, the screens in use and their general output were rarely published and this has led to the misperception in much recent literature that little such directed screening was done. Unfortunately, this chapter will not be full of revelation of those methods, though an effort has been made to discuss the types of phenotypic screens that were based on or reflected screens for mutants, early reporter screens, and the mindset that was involved in developing these screens. The question of low output, compared to the riches of the earlier empirical efforts, will be more subjectively dealt with at the end of the chapter.

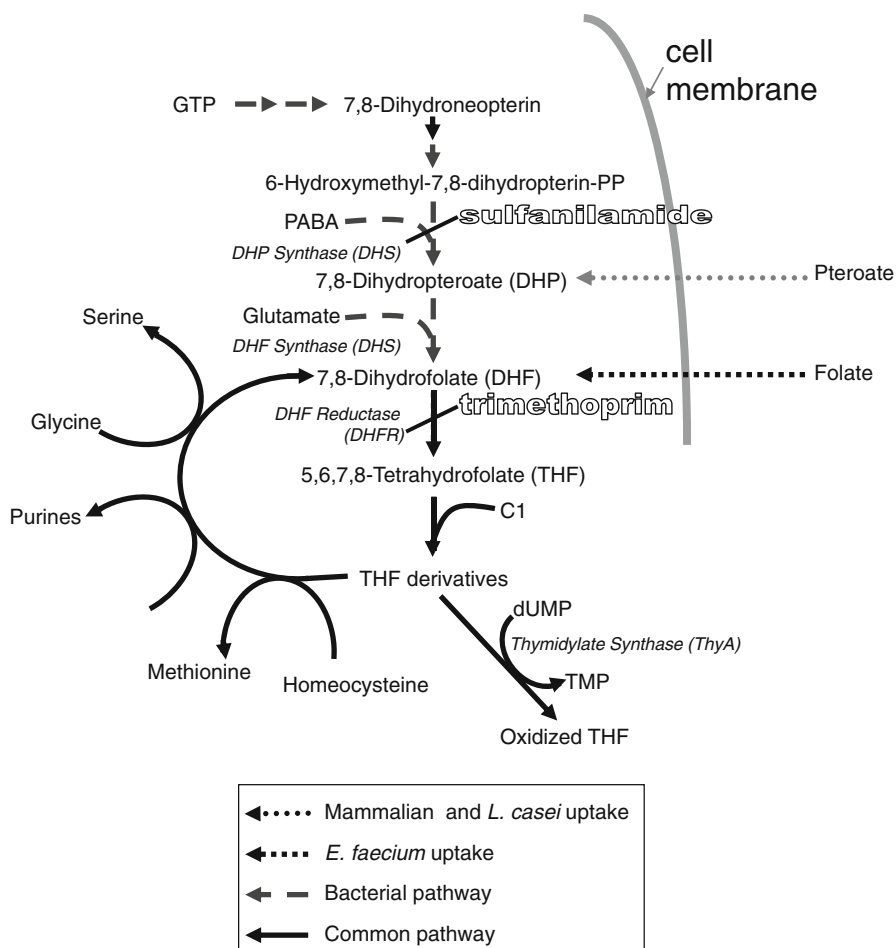
## 2.2 Antibacterial Chemotherapeutics: Antimetabolites Versus Enzyme Inhibitors

### 2.2.1 *Prontosil*

The “magic bullet” of Ehrlich (Nobel Prize in 1908) was the idealized agent that kills the infecting organism while preserving the host. Ehrlich’s work on syphilis involved the systematic synthesis of chemical variants of dyes that selectively stained spirochetes but not host cells and their testing in an animal model; this eventually yielded a (relatively) selective agent, salvarsan, that cured mice and men of syphilis (Fig. 2.1). Working with other dyes, Domagk (Nobel Prize 1939) discovered prontosil rubrum (Fig. 2.1), an azo-dye manufactured by IG Farben, that was active in curing mice of streptococcal infection in vivo but had no in vitro activity [41]. Prontosil provided the first truly selective antibacterial therapeutic with broad



**Fig. 2.1** Salvarsan and inhibitors of the Folate pathway



**Fig. 2.2 Folate pathway.** The general de novo pathway of folate synthesis is shown. While most bacteria cannot take up pterate or folate, mammals and *L. casei* can take up and incorporate pterate into 7, 8-dihydropteroate; *E. faecium* can take up and incorporate folate into 7, 8-dihydrofolate

usage. Later work showed that the activity was due to a metabolite, “prontosil album,” shorn of its red chromophore, identified as para-aminobenzenesulphonamide [33], known as sulfanilamide (Fig. 2.1). Fildes had hypothesized that certain disinfectant and other agents inhibited bacterial growth by interfering with substances essential for the growth of the organism [52] and at his suggestion, Woods investigated the interference by an unknown factor in yeast extract with the activity of sulfanilamide, finding that the substance was likely to be para-aminobenzoic acid (PABA, Fig. 2.1) [196]. This led Woods to postulate that sulfanilamide was active due to its resemblance to PABA, which enabled its competition with PABA for an essential anabolic enzyme. At the time, the role of PABA in bacterial (or any) metabolism was unknown. It was soon shown to be critical in the folate pathway (Fig. 2.2), which provides intermediates to a number of metabolic pathways. This is,

essentially, the basis for rational chemotherapy hypothesized by Fildes and Woods, the design and selection of compounds that resemble true metabolites. But how does the competition of sulfanilamide with PABA explain the selective toxicity of sulfanilamide? Would other such antimetabolites show species selectivity?

The idea of selective toxicity was addressed by Work whose main thesis was that selective toxicity could only be approached rationally if the basis for selectivity between host and infecting organism was understood [197]. He argued that even when antibacterials showed excellent selective toxicity and also appeared to inhibit formation of a specific cellular molecule, it could not be said that the selectivity is due to species specificity in the synthesis of that molecule without an understanding of the characteristics of the synthesis that define species specificity.

Indeed, in the case of sulfanilamide, selectivity is based on the fact that most bacteria cannot take up folates but must endogenously synthesize dihydrofolate via several steps including the synthesis of dihydropteroate from PABA and 6-hydroxymethyl-7, 8-dihydropterin-PP via dihydropteroate synthase (DHS), while humans cannot synthesize folate but must take it up from exogenous sources (Fig. 2.2). Thus the antimetabolite concept is not any insurance of selectivity.

## 2.2.2 Trimethoprim

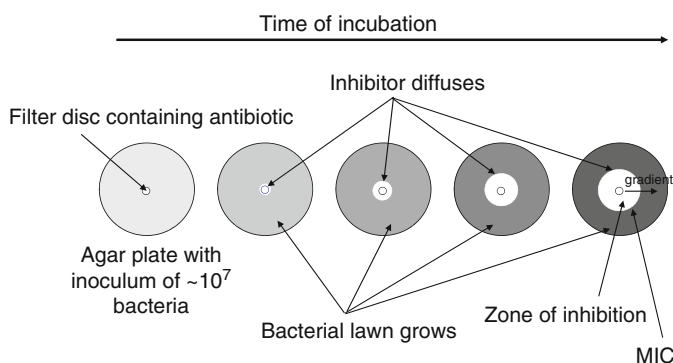
The approach to chemotherapy via rational design and screening for antimetabolites was explored at Wellcome by Hitchings, Elion (Nobel Prize for their efforts in 1988), and their coworkers, who made great strides in anticancer, antiparasite, and antibacterial chemotherapy in part through investigation of folate antagonists. Initially working with *Lactobacillus casei*, a bacterium that *can* utilize exogenous folate (Fig. 2.2), Hitchings and coworkers recognized that folate utilization was competitively inhibited by nearly all 2, 4-diaminopyrimidines (Fig. 2.1) [72]. However, they recognized that the competitors showed more tissue and species specificity in this competition than did 4-amino analogs of folate and so might not be acting as simple antimetabolites. The target of the inhibitors was not recognized until the later steps in the pathway were elucidated (Fig. 2.2), and it was shown that the 2, 4-diaminopyrimidines blocked the reduction of dihydrofolate to tetrahydrofolate by the enzyme dihydrofolate reductase (DHFR), an enzyme present in bacteria and humans [73, 74]. Once the basis for specificity and selectivity was understood to reside at the level of enzyme interaction and cellular uptake mechanism, a more rational approach to design and testing of analogs could be undertaken.

Trimethoprim (2, 4-diamino-5-[3,4,5-trimethoxybenzyl]pyrimidine, Fig. 2.1) was the result of this effort, its final choice based on its tolerability in monkeys and excellent antibacterial potency [27]. Additionally, it was shown that trimethoprim and sulfanilamide were synergistic in their action by the use of in vitro methods [27, 48] by dint of their double blockade of the folate pathway.

As will be discussed below (Sect. 6.1), antifolate screening among natural products was undertaken at Fujisawa [136] using a phenotypic whole cell screen.

## 2.3 Natural Product Screening

The earliest antibacterial discovery – salvarsan, sulfas, trimethoprim – focused on synthetic chemicals, but the “golden age” was one of natural product screening. The work of Fleming (Nobel Prize 1945), Waksman (Nobel Prize 1952), and others has been much reviewed and is covered in the previous chapter. The screening of fermentation broths of Actinomycetes yielded a variety of antibiotics that were relatively quickly developed for clinical use starting in the 1940s. The general screening method was an agar diffusion assay in which fermentation samples were usually applied to filter paper discs that were placed on an agar plate inoculated with a bacterial culture (Fig. 2.3). By the 1950s, the rate of detection of novel compounds had declined. As reviewed by Baltz [10], work at Merck and Lilly in the 1950s showed that between 12.5% and 25% of randomly isolated Actinomycetes produced antibacterial antibiotics and that between 10,000 and 20,000 cultures had to be screened to yield about 10 novel compounds. That is, novel products were found in 0.1% of cultures and any specific novel compound might be found at a frequency of  $\sim 10^{-4}$  per culture screened. Clinical candidates were found among novel compounds at a frequency of 2–10%. As more cultures were screened and more “knowns” accumulated, the frequency of any specific novel compound being found decreased to between  $10^{-6}$  and  $10^{-7}$  per culture by 1976. This rarity is exacerbated by the high prevalence of very common compounds (such as streptothricin, actinomycin, streptomycin, tetracycline), in addition to the accretion of the relatively lesser known compounds over time. Clearly, random screening for inhibitors of bacterial growth followed by unprioritized isolation of activities had become impossible early on in



**Fig. 2.3** *Agar diffusion assay for antibiotic activity.* Petri dishes are filled with a thin layer of agar containing growth medium. Bacteria ( $\sim 10^7$ ) may be inoculated directly in the molten agar (when sufficiently cooled) or spread on top of the hardened agar. Samples are applied to filter paper discs on the agar or into wells cut in the agar. As the culture grows and any antibiotic on the disc diffuses into the agar, a zone of inhibition (ZOI) is formed around discs containing antibiotic. The size of the ZOI is a function of the concentration of antibiotic on the disc, the diffusion rate of the antibiotic compound at the incubation temperature in the given medium, and the growth rate of the bacterial strain. The outer edge of the ZOI lies at the effective minimal inhibitory concentration (MIC)

the history of antibiotic discovery. Indeed, prioritization was required. The methods used to distinguish novel compounds from known compounds at the earliest stage possible in the discovery process are known collectively as “dereplication.”

### 2.3.1 *Dereplication*

Various dereplication methods of classifying fermentation broths as containing compounds “already seen” have been employed in order to reduce the number of “hits” from a screening system requiring time consuming chemical isolation. Importantly, it is necessary to track the biological (antibiotic) activity as well as the chemical entity during dereplication [162]. For chemical classification or identification sufficient to recognize whether an activity is novel or previously seen, it is often necessary to follow the activity through several, usually orthogonal, fractionation steps, in order to correlate the biological activity with the chemical signature. Potent activities present in small quantities may be practically chemically invisible in the face of more major components.

The chemical fractionation and isolation techniques used to track biological activity to a specific chemical entity have evolved over time, as have methods for identification of chemical structure. Biological methods for identifying, or at least classifying activities as common or potentially novel with minimal need for fractionation have also evolved. One such method is the use of a panel of bacterial isolates that have been selected as resistant to specific common compounds. This method was proposed by Stansly [167] and put into practice by Stapley [168]. Selected fermentation broths are tested for their antibacterial activity on the panel of resistant isolates; if the broth contains a compound to which one of the test panel is resistant, then the broth may be classified as containing a known or at least a compound cross-resistant with a known. This type of panel for dereplication and identification has been employed and refined over time with the use of a broad spectrum of bacteria, often under varying media or growth conditions or in the presence of specific  $\beta$ -lactamases, giving patterns of sensitivity characteristic of specific compounds. In large part, the differences among “wild type” bacteria in their innate sensitivity to antibiotics is based on their permeability to the compound much more so than to the presence or absence of a given target. In any case, by creating a large “deck” of results from a panel of organisms, patterns can be recognized that indicate the presence of a previously seen compound. But can that find novelty?

Since many, if not most, Actinomycete broths contain multiple antibiotics (or at least, the isolates have the capacity to make multiple antibiotics), the patterns seen in biological dereplication panels may reflect mixtures. Such mixtures may give seemingly unique novel patterns that turn out, upon fractionation, to resolve into combinations of knowns. Even with mixtures, if there are enough key organisms (resistant to specific compounds) and specific signatures, most broths will be classified as

non-unique (i.e. as containing mixtures of knowns). However, as noted above, one must dereplicate both chemical and biological activity. The biological dereplication tool may identify major knowns in a mixture, but it may miss novel minor components. Thus, while chemical and biological dereplication tools are available, they are inefficient for finding novelty among empirically screened antibiotic activities selected from tens of thousands of fermentations of randomly isolated organisms. Underlining the need to avoid common randomly selected organisms for screening, Baltz [10] has reasonably posited that the rare compounds sought are those for which the producing machinery has most recently evolved while the commonly seen antibiotics are older and their producers more widespread. Thus, groups engaged in natural product screening have made great efforts over the years to include uncommon producing organisms, exploit remote ecological niches, and use novel isolation methods and selective media to raise the likelihood of finding the rare producers of novel compounds. While those rare producers may be more prevalent among screened organisms, the likelihood is still great that the bulk of antibiotic activities seen will be common and still require dereplication. As a focus on rare organisms alone did not solve the problem, the primary screening process itself evolved from empirical “kill-the-bug” screens to more directed, “rational” screening.

### 2.3.2 *Screening as a Means of Dereplication*

An intrinsic problem of empirical screening is the ease with which gram-positive organisms such as *S. aureus* and *B. subtilis* are killed by common natural products and members of synthetic chemical libraries. Gram negatives are more resistant to large and hydrophobic compounds due to the inherent selectivity of their two bounding membranes and potentiated efflux mechanisms [160]. Thus, one could choose to use gram negatives for high throughput empirical screening such as a multiply resistant *E. coli* strain as recommended by Baltz [11, 12]. It is true that this will eliminate the few gram positive-specific molecular targets and the novel compounds to which the screening organism is impermeable (but which might provide a lead for chemical modification to improve spectrum), but this approach provides both selectivity and a degree of dereplication or elimination of the common knowns (to which resistance can be obtained). In fact, much of the early screening (after Waksman’s initial screening for antimycobacterials) was directed toward broad spectrum or gram-negative activities. When novel compounds with solely gram-positive spectra were discovered, they were often directed toward animal health and animal growth promotion.

While retrospective accounts of early target directed screening may emphasize the choice of target [54, 56, 134], it is clear that choosing to evaluate a subset of antibiotics, selected by some practical or rational criterion, has the benefit of reducing the amount of dereplication to be done. One need only ask if this new thing is

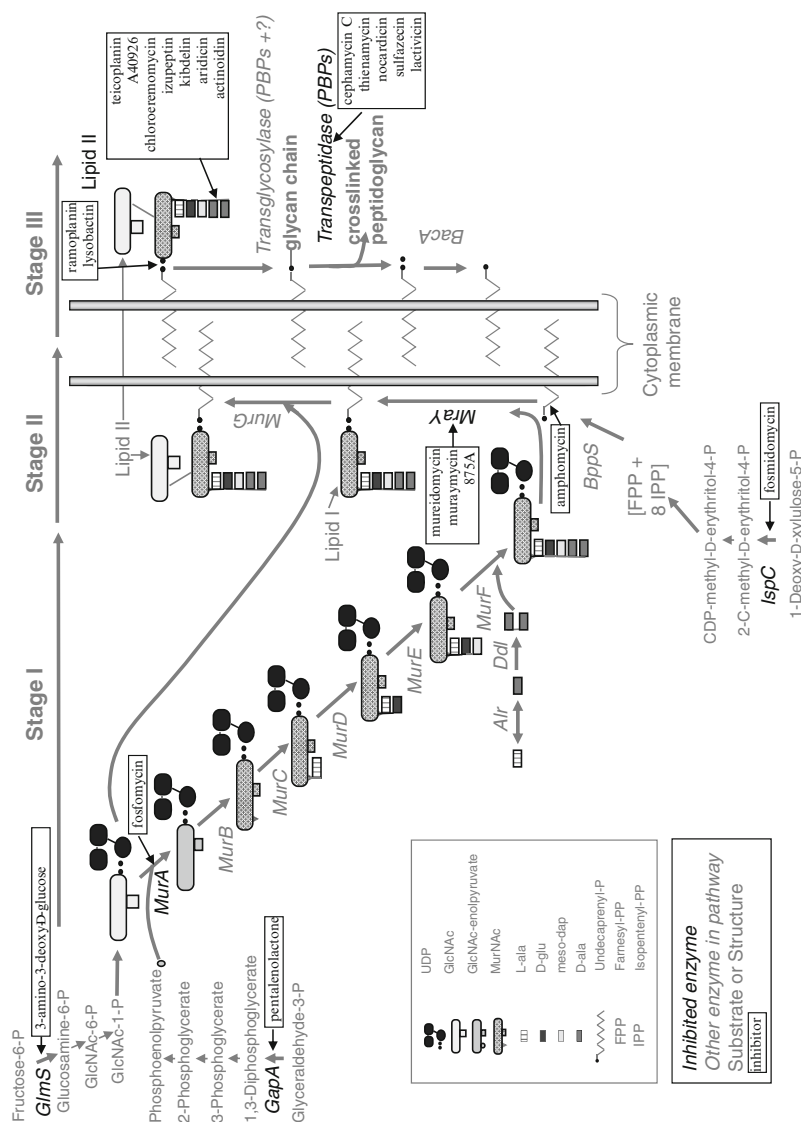
like the finite number of other things acting in a similar way. Thus, screening for inhibitors of a specific pathway or with a particular selective screen is a method, in itself, of dereplication. This has been termed targeted dereplication and is illustrated in screening for HIV inhibitors [161].

The  $\beta$ -lactam and glycopeptide antibiotics found in empirical screening had proved to be efficacious and display high selectivity and low toxicity, because they inhibit the bacterial specific peptidoglycan synthesis pathway. The other major classes of antibiotics found early on targeted protein synthesis and the basis for selective toxicity of those was not as clear at the time. Indeed, many, if not most, antibacterial protein synthesis inhibitors also inhibit mitochondrial protein synthesis but maintain selectivity most likely on the basis of selective permeability, or short-term use. Furthermore, as will be seen in the following section, the study of cell wall inhibitors had revealed a number of characteristic phenomena that could be exploited in screening. Thus, a large number of screens for cell wall inhibitors were devised and run starting in the 1960s, combining the rational choice of a desirable target pathway and the opportunity for dereplicating natural products by narrowing the range of active compounds to which an unknown must be compared in order to predict novelty at an early stage after detection.

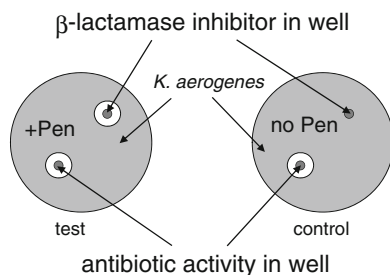
## 2.4 Rational Screening for Inhibitors of Cell Wall Synthesis

The recognition of mechanism of action of antibiotics and antibacterial chemotherapeutic agents began with the concept of antimetabolites and continued with the discoveries of antibiotics. For penicillin, the mechanism of action was delineated via both biochemical and morphological means [106, 141, 170]. Park's discovery of the nucleotide-linked peptide intermediate of cell wall synthesis (UDP-GlcNAc-L-alanine-D-glutamate-L-lysine-D-alanine-D-alanine, called "Park nucleotide") that accumulated during penicillin treatment of *S. aureus* was a key finding in the understanding of the peptidoglycan synthesis pathway [141]. Thus penicillin, and later other antibiotics, notably protein synthesis inhibitors, proved useful tools for studying bacterial physiology in dissecting the basic pathways of macromolecular synthesis in bacteria. The pathway of cell wall synthesis (in *E. coli*) is shown in Fig. 2.4. The first committed step is catalyzed by MurA, starting the *mur* cascade of cytoplasmically located steps that are sequentially required for the synthesis of Park nucleotide which is translocated to the undecaprenol-P carrier lipid by MraY to form Lipid I on the inner surface of the cytoplasmic membrane. GlcNAc is transferred to Lipid I by MurG to form Lipid II, which is transported to the outer surface of the cytoplasmic membrane. The disaccharide-pentapeptide of Lipid II is joined to existing chains of disaccharides by transglycosylase (a function mainly of the dual function large penicillin binding protein, PBP1b) and the peptide chains are cross-linked by the transpeptidase activity of several different PBPs. The undecaprenyl-P is recycled to the inner face of the cytoplasmic membrane.





**Fig. 2.4** *Cell wall pathway and inhibitors.* The committed steps of the peptidoglycan synthesis pathway of *E. coli* are shown, from MurA through transpeptidation by PBPs. Additionally, parts of pathways providing substrates for the cell wall pathway, N-acetyl-glucosamine (GlcNAc), undecaprenyl-P, and phosphoenolpyruvate are indicated. Inhibitors discovered through cell wall pathway-directed screening are noted in *boxes* (This figure is adapted with permission [158])



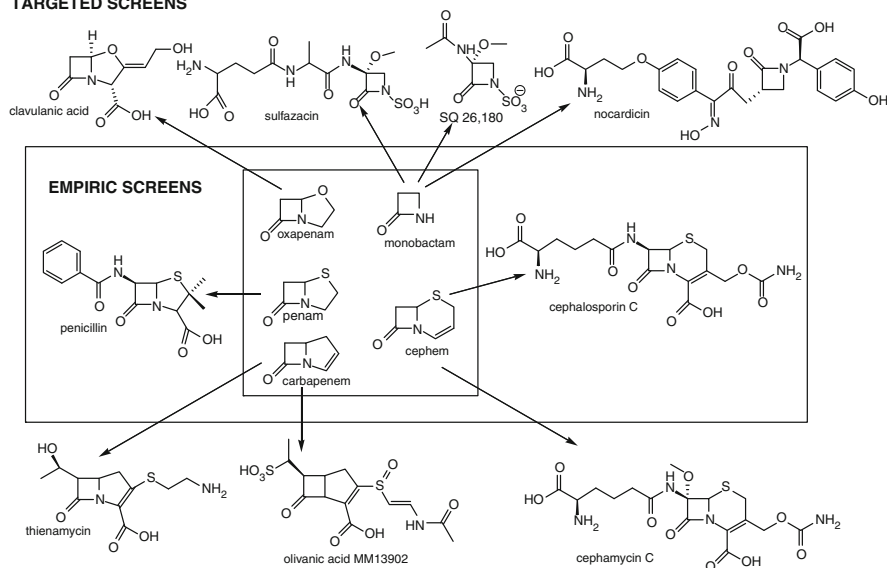
**Fig. 2.5** Beecham “KAG” assay for  $\beta$ -lactamase inhibitors. As described in the text, blood agar plates are prepared with one of each pair containing benzylpenicillin. The plates are seeded with *Klebsiella aerogenes* producing a  $\beta$ -lactamase that renders it insensitive to the penicillin; samples containing  $\beta$ -lactamase inhibitor candidates are deposited in wells in the paired plates (i.e., the same sample is applied to both of each pair of plates). During incubation, the bacterial lawn grows and any  $\beta$ -lactamase inhibitor in the wells diffuses and inhibits the  $\beta$ -lactamase produced by the bacterial inoculum, thus rendering the cells sensitive to the penicillin in the plate. Hence, a zone of inhibition (ZOI) will be formed on the penicillin-containing plate around wells containing such  $\beta$ -lactamase inhibitors. Wells containing antibiotic activity will yield ZOI on plates both with and without penicillin

### 2.4.1 $\beta$ -Lactamase Inhibitors

Soon after the introduction of penicillin into the clinic, resistance, due to degradative enzymes, the  $\beta$ -lactamases, became evident [125]. Interestingly, these enzymes were found to have been present in pathogens, dating from long before the introduction of penicillin [14, 15]. Accordingly, some of the earliest rational approaches to antibacterial therapeutic discovery among natural products concerned the search for inhibitors of  $\beta$ -lactamases. The methods by which the  $\beta$ -lactamase inhibitors were discovered have been noted and reviewed by various authors [23, 75], including those involved in the discoveries, but, since the screening procedures were often published after disclosure of compounds, the timing of the discoveries is not always clear.

First published in 1976 [25], although reported to have been used as early as 1967 [23], the KAG screen of Beecham (Fig. 2.5) employed a blood agar plate containing benzylpenicillin seeded with a lawn of *Klebsiella aerogenes* resistant to penicillin by dint of its production of a Class A  $\beta$ -lactamase. Fermentation samples were inoculated into wells cut in the agar and plates incubated overnight. Diffusible  $\beta$ -lactamase inhibitors would produce a zone of inhibition due to protection of penicillin from degradation by the lactamase. A control plate without penicillin in the agar served to counterscreen against samples producing antibiotics. A differential between the control and test plate was taken to indicate the presence of a  $\beta$ -lactamase inhibitor. Clavulanic acid, an oxapenam (Fig. 2.6), was discovered at Beecham [147] using the KAG assay [25] in an investigation of secondary metabolites produced by *S. clavulagirus*, which had already been described as producing several cephalosporin C related compounds, including cephamycin C, and penicillin N. Clavulanic acid was very successfully developed and introduced in 1981 by Beecham for use in combination with amoxicillin (Augmentin, Co-amoxiclav). Augmentin is generally used orally, although there is a parenteral formulation.

## TARGETED SCREENS



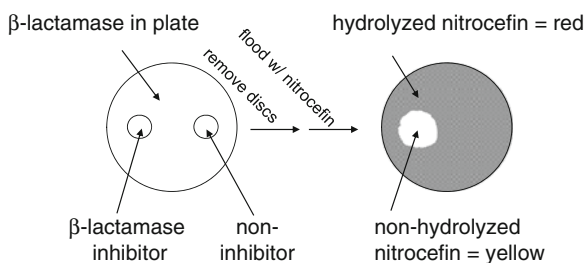
**Fig. 2.6**  $\beta$ -lactams from nature. Structures the inner box illustrate the basic  $\beta$ -lactam platforms. Middle-box structures were found through empiric screening. Structures in the outer box were found through directed screening

Of the commercially available  $\beta$ -lactamase inhibitors, clavulanic acid is the only one that is orally bioavailable.

Olivanic acids (Fig. 2.6), sulfated carbapenem derivatives, were also discovered by Beecham [24, 28] from *S. olivaceus* apparently by use of the KAG screen [23]. The discovery of two  $\beta$ -lactamase-inhibitory compounds from *S. fulvoviridis* had been reported earlier by Umezawa and coworkers [187], one of which, MC696-SY2-A, was later shown [112] to be an olivanic acid (equivalent in structure to MM 4450 of Beecham). It is likely that an agar diffusion assay described in the discovery paper [187] was used for screening. *S. aureus* was used as an indicator strain and agar plates were prepared containing appropriate amounts of penicillinase, penicillin and the test organism. Paper discs containing an inhibitor were applied and the plates incubated overnight. Since the penicillinase to penicillin ratio was adjusted so as to leave no undigested penicillin, the diffusion of inhibitor from the paper discs would thus lead to the formation of a zone of inhibition on the test strain due to protection of the penicillin.

A screen for  $\beta$ -lactamase inhibitors was published by Squibb in their disclosure of a novel, potent non- $\beta$ -lactam inhibitor of  $\beta$ -lactamase, izumenolide (EM4615, Fig. 2.7) [26, 110]. In this screen (Fig. 2.8), fermentation samples were added to 11 mm cellulose discs, the discs dried and applied to agar plates containing the Type A  $\beta$ -lactamase TEM-2. Plates were incubated at 37°C for 3 h, the discs removed and the plates flooded with a solution of a chromogenic cephalosporin (presumably nitrocefin [173]) that turns red upon hydrolysis of the  $\beta$ -lactam ring. Thus, a positive response in the assay is a light yellow zone (of non-hydrolyzed nitrocefin) around the site of sample application against a lawn of red (hydrolyzed nitrocefin).





**Fig. 2.8** Squibb assay for  $\beta$ -lactamase inhibitors. Plates containing  $\beta$ -lactamase in agar are prepared and 11 mm filter discs containing  $\beta$ -lactamase-candidate samples are distributed on the agar. Plates are incubated for 3 h at 37°C, then discs removed and the plates flooded with a solution of nitrocefin. In the absence of a  $\beta$ -lactamase inhibitor, the  $\beta$ -lactamase in the plate digests the nitrocefin, yielding the red hydrolytic product. If a  $\beta$ -lactamase inhibitor diffuses from the disc, hydrolysis is prevented, and the nitrocefin retains its native yellow color

The Smith Kline group, also using nitrocefin as a substrate, published their screen for  $\beta$ -lactamase inhibitors [188]. A culture of *K. pneumoniae* 1200 (an isolate containing a  $\beta$ -lactamase, chosen after testing of a variety of strains for their behavior with the positive and negative controls) was grown overnight in a relatively colorless medium and distributed into small test tubes followed by addition of 0.05 ml aliquots of test inhibitors and mixing. No inhibitor was added to negative control tubes. After 5 min incubation at room temperature, one drop of a 0.05% nitrocefin solution was added. Red color (due to hydrolysis of nitrocefin) developed rapidly in negative control tubes but not in tubes containing the positive control clavulanic acid. No screening results were indicated; it is likely that this assay was used for optimization of synthetic or semi-synthetic  $\beta$ -lactamase inhibitors.

In addition to clavulanic acid, the successfully developed  $\beta$ -lactamase inhibitors, sulbactam and tazobactam, are semi-synthetic penam sulfones designed and synthesized at Pfizer [49] and Taiho [7], respectively. Sulbactam is used parenterally most often in combination with ampicillin (Pfizer's Unasyn), and tazobactam was developed by Wyeth in combination with piperacillin (Zosyn).

### 2.4.2 Screens for Spheroplast Formation

In 1992, two reviews appeared that summarized some of the screening strategies for cell wall and other antibiotics that had been used during the 1960s through 1990s, principally at Fujisawa [134] and at Merck [56]. While the Fujisawa screens had been noted previously (see Sect. 2.4.3.), the Merck review was the first disclosure that a spheroplasting method had been used as a primary screen (SPHERO) at Merck for 30 years. As noted in the Gadebusch review, "By the early 1960s it was apparent that a deliberate search for cell wall antibiotics was warranted based on considerable evidence of their efficacy and unusually low toxicity" As early as 1962, Dr. Eugene Dulaney at Merck "set about performing detection assays using the

physical effects produced by cell wall-active agents as the clue for the detection of such microbial products” [56]. It is both fitting and frustrating to note Dr. Dulaney’s contribution here, as he was this author’s greatly esteemed mentor in the art and science of antibacterial screening but, throughout his long career, he never published on any of the screens, including SPHERO, that he devised.

Details of the relative robustness (false positives, reproducibility, throughput) of the SPHERO screen have not been disclosed but it is known [56, 169] that conditions for spheroplasting were based on the findings of Lederberg [106]. In an effort to find conditions for studying protoplasts of gram negative bacteria, Lederberg observed that protoplasts (later called spheroplasts in gram negatives) were formed upon treatment of *S. typhimurium* and *E. coli* with penicillin in the presence of sucrose and  $Mg^{++}$  under conditions which supported growth. At Merck, samples, generally clarified natural product broths or extracts, were added to bacteria in osmotically stabilized medium and cell morphology was observed by direct microscopy after a period of several hours. Spheroplasts appear as large round refractile bodies. As inhibition of mass increase will prevent the spheroplasting action of penicillin, such a screen would be interfered with by the presence of agents inhibiting certain other cellular functions such as RNA or protein synthesis. Similarly, the presence of membrane active agents could lead to lysis of any spheroplasts formed. As natural product broths often contain a mixture of antibacterial substances, this can be problematic. One way of overcoming it is to run several dilutions or to grow isolates in several media and conditions in the hopes that mixtures will be made in or diluted to varying ratios so that a positive response may be revealed. Conversely, the fact that the production of spheroplasts requires that the cell’s mass increase is not compromised, the positives that are found in such screens are less likely to be toxic or have multiple mechanisms of action. Once activities were detected in the SPHERO primary screen, dereplication could be undertaken. This included determination of the bacterial spectrum and relative potency of spheroplasting activity of the unknown active, its sensitivity to various  $\beta$ -lactamases and other tests of antibacterial spectra to help distinguish old true positives from novel activities, as discussed above (Sect. 2.3.1).

The SPHERO screen discovered fosfomycin, cephamycin C, and thienamycin as well as other carbapenems (epithienamycins) and the uracil containing antibiotics, A859A and 875A, likely inhibitors of *MraY*. Ensanchomycin and prenomycin, compounds related to the transglycosylase inhibitor, moenomycin were also discovered in SPHERO. Pentalenolactone (Fig. 2.7) and many previously seen compounds, including cycloserine (inhibitor of alanine racemase (*Alr*) and D-ala-D-ala ligase (*Ddl*) (see Fig. 2.4) were also detected as spheroplast-formers [56, 169].

Fosfomycin (Fig. 2.7), a broad-spectrum phosphonate antibiotic produced by several streptomycetes [71], targets *MurA* [88], the first committed step in the peptidoglycan pathway (see Fig. 2.4). It was developed in Europe by CEPA, Madrid, which had collaborated with Merck in its discovery.

Cephamycin C and thienamycin, both  $\beta$ -lactams (Fig. 2.6), became the subject of extensive chemical programs. Cephamycin C [169], also discovered by Lilly in the course of examining known *Streptomyces* producers of penicillin [124], was the first cephem discovered that is produced by bacterial sources, including *S. lactam-durans*. It is highly resistant to many  $\beta$ -lactamases and has an almost exclusive

gram-negative spectrum that was broadened through chemical modification, yielding the semi-synthetic cefoxitin [94]. Cefoxitin (Mefoxin) has been a highly successful parenteral compound.

Thienamycin (Fig. 2.6), the first carbapenem discovered, has an extremely potent broad antibacterial spectrum including *P. aeruginosa* and *S. aureus*, and was isolated from *S. cattleya* (which also produces Cephamycin C) [1, 90, 91]. The compound proved unstable and, although the subject of extensive fermentation improvement and chemical isolations studies [183], difficult to ferment in commercial amounts. Stability was much improved by use of the amidine derivative, N-formimidoyl-thienamycin, or imipenem, and a multi-step synthetic route worked out for its commercial production [120, 150]. Imipenem's lability to a human renal degradative enzyme, dehydropeptidase I, was countered by discovery of an inhibitor, cilastatin [62], that could be given in combination with imipenem to preserve its activity [89]. The combination drug, Primaxin (Tienam) has been a staple in the ICU since its introduction in the late 1980s.

It is clear that the ability of compounds to form spheroplasts was a part of the screening and characterization system at Sankyo, as there is a series of papers noting discovery of a number of compounds with spheroplast forming activity. But it is not clear that spheroplasting was run as a primary screen (as it was at Merck); this is not an empty difference. The nature of the primary screen run, its hit rate, false positive rate, reproducibility, and sensitivity are all critical to the success of any screening program, especially a natural products screening program depending upon fresh fermentation broths or extracts (as most of these were) where downstream sample availability, handling, purification and identification are all labor and resource intensive. Spheroplast-forming antibiotics discovered by workers at Sankyo include mureidomycin [83], globomycin [79], malioxamycin, pentalenolactone and iso-U-22956 [176], and fosfonochlorin (a fungal product) [177] (Fig. 2.7). Mureidomycin has been demonstrated to be an inhibitor of peptidoglycan synthesis, specifically an inhibitor of *MraY* [82]. Fosfonochlorin selectively inhibits incorporation of diaminopimelic acid (DAP) into cell wall of *E. coli* over arginine into protein but its mechanism is unknown. Malioxamycin is a weak gram-negative antibiotic that preferentially inhibits DAP incorporation into cell wall but also inhibits protein synthesis to some extent. No mechanism for iso-U-22956 or its previously discovered isomer, U-22956 [116], have been proposed. The mechanisms of action of pentalenolactone and globomycin and their relation to spheroplast formation are discussed below (Sect. 2.4.2.1).

#### 2.4.2.1 Mechanisms of Action of Spheroplasting Compounds Acting Outside the Committed Steps of the Cell Wall Pathway

While the spheroplasting screen and other cell wall screens, certainly detected inhibitors of peptidoglycan synthesis, they also detected activities that were unexpected. They are mentioned here to emphasize the place of serendipity in the discovery process and to underline the useful “fuzziness” of such assays. For natural product screening, where the goal is to find novelty and selectivity, it is not critical that the hits be “on target” but that the screen is robust and turns up interesting compounds.

## Fosmidomycin and Other Phosphonates

Workers at Fujisawa, discovered a number of phosphonate compounds containing an N-acylhydroxamino function produced by several Streptomyces by screening for activity against a nocardicin supersensitive *P. aeruginosa* strain. All of these, including fosmidomycin (FR-31564, Fig. 2.7), FR-900098, FR-32863, FR-33289 [103, 135], yielded spheroplasts in hypertonic medium and were thus thought to inhibit cell wall synthesis. They were later found to inhibit synthesis of menaquinones via the non-mevalonate (MEP) isopentenyl-diphosphate biosynthetic pathway present in many bacteria, plants and parasites but not mammals [153, 182]. Fosmidomycin and FR-900098 are specific inhibitors of 2-C-methyl-D-erythritol 4-phosphate synthase (IspC) the first committed step in the non-mevalonate pathway [104]. Fosmidomycin is synergistic with cell wall and some other inhibitors [126]. It is likely that spheroplasts are formed by fosmidomycin because in bacteria using the non-mevalonate pathway, undecaprenyl-P, the cell wall carrier lipid, is a product of the MEP pathway (Fig. 2.4). The MEP pathway appears to be a reasonable antibacterial target and a whole cell phenotypic screen for such inhibitors has been described [182]. Both fosmidomycin and FR-90098 have shown oral efficacy in mouse *Plasmodium vinckei* (a rodent malaria parasite) infection [84]. In humans, rapid reduction in *P. falciparum* parasitemia was seen in many subjects but recrudescence was common [107]. Studies of clindamycin-fosmidomycin combinations appear more promising but more trials are needed [22].

## Pentalenolactone

Pentalenolactone (Fig. 2.7), also called arenaemycin, was reported to form spheroplasts in the Sankyo screen [176], was active in many of the cell wall screens run at Merck and was shown to synergize fosfomycin [43], as do many inhibitors of peptidoglycan synthesis. It is a specific inhibitor of glyceraldehyde 3-phosphate-dehydrogenase (GapA), an enzyme in the glycolytic pathway, and required for the synthesis of phosphoenolpyruvate, a substrate of MurA (Fig. 2.4). Bacterial resistance to pentalenolactone is mediated by altered glyceraldehyde-3-phosphate dehydrogenase, as demonstrated in the producing organism, *S. arenae* [55], but the target is not selective for bacteria. A fungal product, heptedelic acid (also known as koniginic acid), has the same target.

## Globomycin

Globomycin (Fig. 2.7), a cyclic peptide antibiotic with a gram-negative spectrum, inhibits signal peptidase II that is specific for the processing of the major lipoprotein of gram negatives, Lpp [76, 81]. Globomycin prevents the release of the prolipoprotein form of Lpp from the cytoplasmic membrane, but it does not prevent the covalent attachment of unprocessed prolipoprotein to the diaminopimelic acid of peptidoglycan; it is this linkage to peptidoglycan that is lethal for globomycin treated cells [198].



Cells lacking Lpp are not killed or spheroplasted by globomycin and incorporation of radiolabeled DAP into cell walls is preferentially inhibited by globomycin at concentrations above its MIC [80]. Thus, it appears that spheroplasting by globomycin is due to the covalent attachment of cytoplasmic membrane and peptidoglycan via unprocessed Lpp, which may ultimately prevent peptidoglycan synthesis.

### 2.4.3 Strains Supersensitive to Cell Wall Active Agents

An approach to screening for cell wall active agents, especially  $\beta$ -lactams, was undertaken independently at several Japanese companies. At Fujisawa, a mutant of *E. coli* derived through N-methyl-N'-nitro-nitrosoguanidine (NTG) mutagenesis was described with greatly increased sensitivity to certain, but not all,  $\beta$ -lactams [4]. Sensitivity was increased 30 to >200 fold to penicillin, cephalosporin C and cephamycin C, but not to cycloserine or fosfomycin. Sensitivity to nocardicin, a *Nocardia* product discovered in this screen, was 400 $\times$  enhanced. Interestingly, mecillinam, which is specific for PBP2, was not more active on the supersensitive strain. It seems likely that the screening strain was, at least in part, a *ponB* (PBP1b) mutant, which could render the strain hypersensitive to  $\beta$ -lactams [172, 178]. Nocardicin [5, 70] was one of (if not the) first natural product monocyclic  $\beta$ -lactams (later called monobactams) discovered in screening (Fig. 2.6).

Takeda scientists [99] isolated a  $\beta$ -lactam supersensitive strain of *P. aeruginosa* through three rounds of NTG mutagenesis and selection. Again, the increased sensitivity appeared to be relatively specific for  $\beta$ -lactams. At least one facet of the increased sensitivity was the loss of the type C  $\beta$ -lactamase, but an additional PBP mutation, most likely PBP1b, is likely to be present. For screening, a four plate agar diffusion assay was used: the parent strain (PS) the supersensitive strain (PSC<sup>ss</sup>), PSC<sup>ss</sup> in the presence of cephalosporinase from *E. cloacae*, and PSC<sup>ss</sup> in the presence of penicillinase from *B. cereus*. The first two plates provided a primary screen for  $\beta$ -lactams while the second two were useful for classification. Thirty thousand strains of fungi, yeasts, bacteria, and actinomycetes were tested with filamentous fungi and actinomycetes yielding the only positives. Thirty-six actinomycetes and 90 fungi produced cephalosporin and/or penicillin N while 25 fungi produced penicillinG type compounds. A number of previously unknown cephalosporins and one penicillin strain were identified in this system. New carbapenems were found [78] in a *Streptomyces* strain. Screening of gram-negative bacteria for activity against  $\beta$ -lactam hypersensitive strains yielded the monocyclic  $\beta$ -lactams sulfazecin and isosulfazecin from a new species of *Pseudomonas* [77]. An interesting non- $\beta$ -lactam compound, lactivicin (Fig. 2.7), was isolated from *Empedobacter lactamgenus* and *Lysobacter albus* and found to bind to essential PBPs [129]. Lactivicin contains N-acetyl-L-cycloserine (interestingly, itself an isooxazolidinone and a  $\gamma$ -lactam) connected via a C-N bond to a furan moiety (see Fig. 2.7). It is sensitive to and induces  $\beta$ -lactamases and, against gram positives such as *B. subtilis*, it behaves solely like a  $\beta$ -lactam. Against *E. coli*, where it behaves as a  $\beta$ -lactam at the MIC, other mechanisms likely to involve inhibition of sulphydryl-containing membrane

proteins are apparent at fivefold the MIC [128]. It is relatively toxic to mice upon parenteral dosing [67].

A  $\beta$ -lactam hypersensitive mutant of *S. aureus* was derived at Otsuka and used in screening, with the reasoning that use of hypersensitive gram negative strains in screening had perhaps biased selection for the more gram negative directed cephalosporins. From among 10,000 actinomycetes and 1,000 eubacteria, 51 carbapenems, 3 cephamycin-C, 5 penicillin-N, 5 fosfomycin and 8 tunicamycin producing strains were found [92]. The high detection rate of carbapenems may justify the initial reasoning.

The Otsuka group also reported a screen using a mutant of *S. aureus* selected for hypersensitivity to D-cycloserine, an inhibitor of alanine racemase (Alr) and D-alala-D-alala ligase (Ddl) [93]. The defect is unclear; however, and testing showed good specificity for specific hypersensitivity to D-cycloserine over all other antibiotics tested. In the screen, besides many instances of D-cycloserine discovered, often from novel producers, O-carbamyl-D-serine, FR-900148 and a novel compound, T-243, were discovered. TA-243 was taken up via peptide transport and subsequently hydrolyzed to aminooxysuccinic acid (Fig. 2.7), an inhibitor of Alr. In fact, aminooxyacetic acid and aminooxysuccinic acid are general inhibitors of pyridoxal enzymes, which include Alr. Accordingly, the antibacterial activity of aminooxyacetate and aminooxysuccinate are reversed by pyridoxal or pyridoxine [176].

The use of strain hypersensitive to other classes of antibiotics have been reported as well (see Sect. 2.5.1)

#### 2.4.4 *Acholeplasma* Screen

In 1979, scientists at the Kitasato Institute published their screening procedure for cell wall inhibitors [137]. The screen was based on the observation that *Mycoplasma* (now considered a genus of the class Mollicutes) such as *Acholeplasma laidlawii* are insensitive to penicillin [6], since they lack a cell wall [115]. The primary screen involved running a sample under two conditions. Samples were selected that inhibited a sensitive wall-containing organism, *B. subtilis*, but not *A. laidlawii*. As the lack of activity against *A. laidlawii* also indicates that the sample is less likely to be membrane-active or generally toxic; this type of screen provides a surrogate for the selective toxicity desired of an antibacterial drug. The Kitasato procedure involved other secondary assays as well. In testing a variety of compounds with known mechanisms of action, it was found that while cell wall active agents did not affect *Acholeplasma*, there were some false positives (i.e., certain compounds with other mechanisms of action also did not inhibit). Thus *B. subtilis* positive, *A. laidlawii* negative samples were tested for their specificity in inhibiting incorporation of radiolabeled precursors into cell wall but not into protein. Finally, activities passing through a membrane filter with a putative size cut-off of 1,000 molecular weight were prioritized for further isolation.

Around 10,000 broth filtrates of fungi, bacteria and actinomycetes were tested in this screening system. One new antibiotic, azureomycin, and six previously discovered compounds were identified. Azureomycin is a glycopeptide of as yet unknown

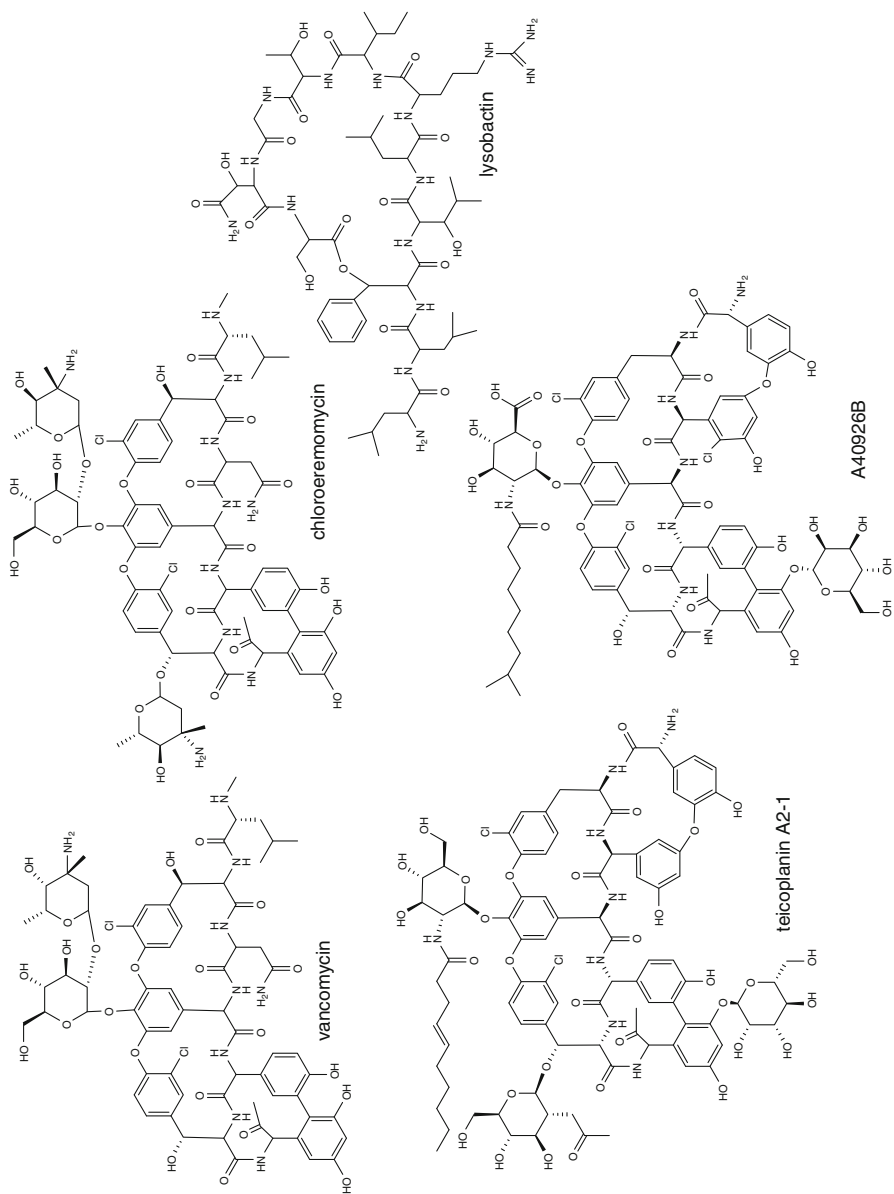
structure, but is likely to act similarly to vancomycin. Of the six previously seen compounds, which included penicillin, cycloserine, ristocetin A and B, amphomycin, and 3-amino-3-deoxy-D-glucose, the mechanisms of action of the latter two had not been previously known.

Amphomycin was shown to inhibit the activity of pentapeptide translocase (MraY; Fig. 2.4) [179, 180], by Ca<sup>++</sup> dependent binding to and sequestration of its substrate, undecaprenyl-P [13]. Amphomycin analogs (friulimicin, MX-2401) have recently been under study as drug candidates [189]; 3-amino-3-deoxy-D-glucose was found to inhibit the formation of glucosamine-6-phosphate from fructose-6-phosphate catalyzed by glucosamine synthetase (GlmS) [181], later recognized as a potential antibacterial target [9, 16] as it is a precursor of the MurA substrate, UDP-GlcNAc (Fig. 2.4). The fact that amphomycin and the ristocetins have molecular weights higher than 1,000 was not entirely unexpected as the MW cutoff of the filter used is not clean. In a later paper, 15 higher molecular weight inhibitors selected in this screen were further examined and the izupeptins, previously unknown glycopeptides, found [164].

Interestingly, a Japanese academic group described a polyether compound laidlomycin, (similar to the ionophore monensin), selected from a screen precisely for its activity against *A. laidlawii*, noting that they were successful in finding novel compounds by this method [98]; this is likely due to exquisite sensitivity of Mycoplasma to certain membrane active agents since they lack the diffusion barrier of the cell wall. Although generally active against mammalian cells, some ionophores have a sufficient therapeutic window for use as coccidiostats in animal health, explaining the interest in screening for such compounds.

### 2.4.5 L-Form Assay

L-forms of bacteria grossly lack cell walls and are bounded simply by the cytoplasmic membrane (although recent work shows that gram negative L-forms do retain a small, essential amount of cell wall material which is required for growth [85]). They can arise “naturally” or through selection in hypertonic medium after treatment with various cell-wall disruptive (e.g., lysozyme) or inhibitory compounds (e.g., penicillin, cycloserine). On solid media they give “fried-egg” colonies resembling those of Mycoplasma. Early workers noted that L-forms were insensitive to inhibitors of cell wall synthesis and sensitive or hypersensitive to other types of antibiotics [38, 59, 87, 195]. Thus, once methodology for reproducible production of relatively stable L-forms was available, their use in screening for cell wall active agents is not surprising. Comparison of L-forms to their parental cell types, rather than Mycoplasma compared to a gram positive wall-bearing bacterium, would likely give a more robust screen, since false positives could arise in the Mycoplasma screen due to differences in non-cell wall targets between the unrelated pairs. Workers at Lepetit used various L-form screens for a number of years in concert with a culture isolation program designed to enrich for Actinoplanes isolates [60, 140]. Teicoplanin (teichomycin A2, Fig. 2.9) [139] and ramoplanin (A-16686, Fig. 2.7) [30] were

**Fig. 2.9** Glycopeptides and Lysobactin

discovered by these methods. In the case of ramoplanin, the screen utilized a methicillin resistant *S. aureus* parent strain to prevent detection of  $\beta$ -lactams [117, 140].

Teicoplanin is a commercially successful parenteral antibiotic used outside of the US. Although the mechanism of action of teicoplanin is the same as that of vancomycin (see below), it is chemically distinct from vancomycin (Fig. 2.9). It has a longer serum half-life and much higher serum binding than does vancomycin and can be given by bolus or IM injection while vancomycin requires slower infusion and multiple daily dosing [44, 123]. Teicoplanin's spectrum is similar to that of vancomycin. It is active against inducible VanB strains of *E. faecalis* where vancomycin is variably inactive, due to failure of teicoplanin to induce VanB expression. As would be expected, constitutive VanB strains are resistant to teicoplanin [50].

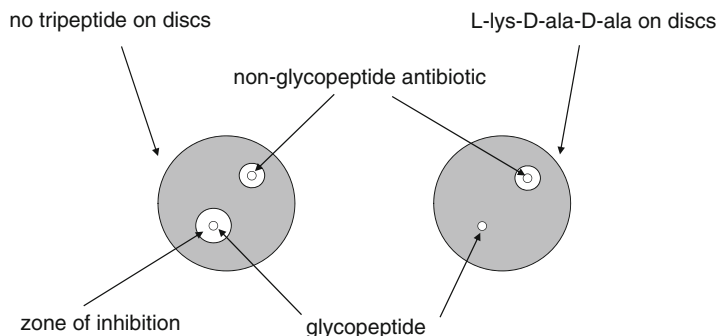
Ramoplanin (Fig. 2.7) is a cyclic glycolipodepsipeptide that binds to both lipid I and lipid II and inhibits enzymes utilizing it as substrate including MurG and transglycosylase (see Fig. 2.4). As it is not likely to enter the cytoplasm, its target in whole cells is most probably the transglycosylase reaction [51, 111]. It has broad gram-positive activity, no oral absorption, and poor parenteral pharmacokinetics. Ramoplanin has been under clinical investigation for use in eliminating intestinal vancomycin resistant enterococci in at risk patients and for *C. difficile* associated diarrhea.

The L-form screen was used at Vicuron (which had absorbed Biosearch Italia, the latter form of Lepetit after its sojourn with Merrel Dow) for the detection of novel lantibiotics [29]

#### **2.4.6 Specific Screens for Novel Glycopeptides Based on Mechanism of Action**

The discovery of the glycopeptide vancomycin was reported by Lilly in 1955 [118] and that of ristocetin by Abbott in 1957 [63]. While their structures were not fully elucidated until much later [68, 69, 192], they were rapidly introduced into the clinic as anti-staphylococcal agents. The mechanism of action of these compounds was initially recognized as inhibition of peptidoglycan synthesis [148, 190] and treatment of sensitive cells with vancomycin was shown to lead to the accumulation of UDP-MurNAc-pentapeptide (Park nucleotide) in *S. aureus*, as does penicillin [3]. Vancomycin and ristocetin were found to bind to cell walls [18, 19] and to various intermediates of peptidoglycan synthesis terminating in D-ala-D-ala [143]. A synthetic peptide, diacetyl- $\alpha\lambda$ -L-diaminobutyryl-D-ala-D-ala, was shown to compete with UDP-MurNAc-L-ala-D-isoglu-m-dap-D-ala-D-ala for vancomycin binding and could reverse growth inhibition by vancomycin of *B. megaterium*, *M. lysodeikticus* and *S. aureus* [127]. Those competition assays formed the basis of later screens for novel glycopeptides. Thus, a number of screening systems for glycopeptides based on their preferential binding to cell wall precursors terminating in D-ala-D-ala were run by several companies.

At Smith Kline, French, a screening system designed to find compounds with the same target-binding site as vancomycin was designed and used in tandem with a



**Fig. 2.10** Tripeptide antagonism screen for glycopeptides. Plates are prepared as for standard agar diffusion (Fig 2.3). Samples are applied in duplicate to filter paper discs, one disc also containing 100  $\mu$ g of L-lys-D-ala-D-ala. In this illustration, discs on the right plate contain the tripeptide. Diffusion of glycopeptides that bind to the tripeptide is severely retarded, preventing the formation of a ZOI

culture selection and medium development program [146]. Two different strategies were used in screening. In the first, a prescreen for differential activity on wild type versus a vancomycin resistant isolate of *S. aureus* was followed by a tripeptide reversal screen. Later, the two screens were run in parallel. The selection of a highly vancomycin resistant derivative (vancomycin MIC >100  $\mu$ g/ml) of a sensitive *S. aureus* strain (vancomycin MIC 1.6  $\mu$ g/ml) consisted of plating 0.1 ml of an overnight culture on Mueller-Hinton agar containing 100  $\mu$ g/ml vancomycin, subjecting the plate to UV irradiation (sufficient to give 99% killing) and incubation of the plate for 40 h at 37°C. Resistant isolates were obtained, which seems somewhat surprising. Use of the resistant organism paired with its parent detected 7 of 11 glycopeptides but also detected 30% of 165 other known antibiotics; additionally, this test was not very sensitive. The motivation to use this singularly non-robust, poorly selective and insensitive test as a primary screen was apparently the initial limited availability of the tripeptide used in the secondary screen.

The tripeptide antagonism screen (Fig. 2.10) consisted of measuring the zone size differential between identical samples on two 6.5-mm paper discs, one of them impregnated with 100  $\mu$ g of diacetyl-L-lys-D-ala-D-ala, applied to a lawn of *B. subtilis*. Control studies showed that 100  $\mu$ g of tripeptide reversed the activity of 3.1  $\mu$ g of vancomycin on the disc. Hence, the zone of inhibition of antibiotic samples containing glycopeptides is strongly reduced around discs containing the tripeptide. The tripeptide reversal screen appeared to be completely selective for glycopeptides. With the vancomycin resistance prescreen, 344 initial hits (14%) out of 2,457 cultures screened ultimately yielded 5 glycopeptide producers (0.2%). With the tripeptide reversal primary screen, 2.9% (57 of 1,936 cultures) were positive of which 41 (2.1% of input cultures) were identified as glycopeptide leads. The 2.9% hit rate is remarkably high and reflects the screening of samples which were prepared from cultures isolated on and fermented in media selected to favor the capacity to produce and the production of glycopeptides. A number of glycopeptide classes were found in this campaign, including the aridicins [163] and the similar kibdelins [152], Actinoidin A2 [40] parvodacin [32] and A42867 [149].

At Merrell Dow (which had subsumed Lepetit), the glycopeptide A40926 (Fig. 2.9) was selected in a screen that involved passage of fermentation samples over agarose- $\epsilon$ -amino-caproyl-D-ala-D-ala [35] and elution of adsorbed glycopeptides with 1% aqueous ammonia at pH 11 [61]. A40926 had been selected for further work on the basis of its activity against *N. gonorrhea*. It is the precursor of dalbavancin, which is currently in development by Pfizer (by way of the re-emergence of the Lepetit unit, then called Biosearch Italia, from Merrell Dow and its the merger with Versicor to form Vicuron which was then acquired by Pfizer). Dalbavancin has a long half-life and an alkyl side-chain like teicoplanin, aridicins, and kibdelins. This resin-dipeptide screen has the advantage over the tripeptide reversal screen run on agar plates of being able to detect glycopeptides in the presence of interfering activities or other antibiotics, as they would have the potential of masking the differential.

Lilly developed a solid state ELISA assay for screening broths [113] using a BSA-linked pentapeptide [ending in D-ala-D-ala] adsorbed to a microplate. Binding of vancomycin covalently linked to alkaline phosphatase could be measured by alkaline phosphatase activity and unknown samples tested for competition with vancomycin for that binding. Lilly discovered eremomycin and chloroeremomycin (Fig. 2.9, part of the A82846 complex), compounds produced by *Nocardia orientalis*, with this screen [64]. Chloroeremomycin was the progenitor of the semi-synthetic lipophilic compound, LY333328, later named oritavancin [34]. Oritavancin has good activity against staphylococci and vancomycin resistant enterococci, due to chloroeremomycin's potential for dimerization that increases its intrinsic potency and to additional mechanisms of action mediated by the synthetic alkyl side chain [189]. Initially a Lilly development candidate, oritavancin was licensed to Intermune and then Targanta which has recently been acquired by The Medicines Company, which appears poised to continue its development. It should be noted that concurrently with Lilly's discovery of A82846, the equivalent compounds, orienticin and deschloroorienticin were discovered at Shionogi with no directed lead screen identified [184], as was eremomycin by scientists at the USSR Academy of Medical Sciences [57].

#### 2.4.7 Competition with Unfractionated Cell Wall Material

In order to find compounds binding to cell wall components, but with specificities different from those of the glycopeptides, a screen was run at Squibb that used competition with unfractionated wall material, rather than the purified tripeptide used in the glycopeptide screens [133]. A preparation of *S. aureus* cell wall material (boiled in 20% TCA, precipitated and treated with trypsin) was used to prepare agar plates and samples were run on plates with and without this murein addition. Cell wall binding agents are expected to give smaller zones on the murein containing plates. Lysobactin (Fig. 2.9), a dibasic depsipeptide antibiotic, produced by *Lysobacter* sp, was discovered in this way [133], as were the janthinocins [132], cyclic depsipeptide lactones which also possess moderate activity against gram-negatives.



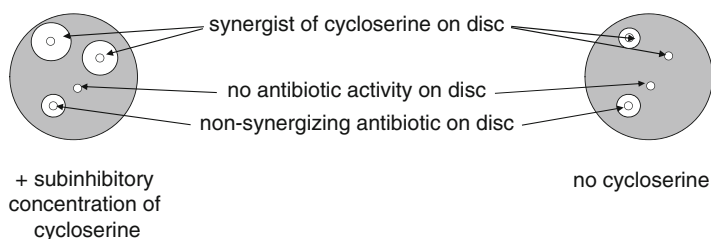
Lysobactin specifically inhibits trace radiolabel into cell wall and not RNA, DNA, or protein at the MIC [21]. Its spectrum is similar to that of vancomycin but with 2–4-fold increased potency and some activity against gram negatives. When the cell wall material was lysozyme digested (thus liberating acyl-D-ala-D-ala), lysobactin was still inactivated but vancomycin was not. Lysobactin thus appears to bind to a different portion of Lipid II than does vancomycin (Fig. 2.4) [21]. Lysobactin treatment does not lead to accumulation of Park nucleotide and causes membrane leakage (of bacteria) above 10-fold the MIC. While the authors suggested that it might be similar to LY146032 (daptomycin), which has some cell wall activity [2] and also depolarizes membranes, lysobactin is structurally more similar to the katanosins and plusbacins discovered at Shionogi [154, 155], which also appear to bind to Lipid II [114]. All of these are quite potent in vivo with apparently reasonable therapeutic indices, but they have not been developed.

#### 2.4.8 $\beta$ -Lactamase Inducers

SQ 26,180 (Fig. 2.6), isolated from *Chromobacterium violaceum*, was the natural product monobactam progenitor of aztreonam [174, 191]. Although the discovery of SQ 26,180 and other eubacterially produced monobactams were initially reported to be based on a supersensitive screen using *Bacillus licheniformis* said to be specific for  $\beta$ -lactams, the screen actually involved sensitive detection of induction of  $\beta$ -lactamase by novel  $\beta$ -lactams [175]. A strain of *B. licheniformis* producing a low titer of  $\beta$ -lactamase was inoculated into agar, plates poured and, after drying, paper discs containing samples were placed on the agar surface and the plates incubated 2–3 h at 37°C. The plates were then overlaid with a solution of nitrocefin that turns red upon hydrolysis with the rapidity of color production dependent upon amount of enzyme. This assay was shown to be 15 times as sensitive as the supersensitive *E. coli* mutant screen of Aoki [4], 200 times more sensitive than microscopic detection of elongated cells of *P. mirabilis* and 3,000 times more sensitive than spheroplast formation in *P. mirabilis*. The only false positives detected in screening eubacteria with the *B. licheniformis* assay were some  $\beta$ -lactones. Squibb discovered a number of monobactams, a carbapenem and cephalosporins produced by bacteria using this screen.

A later screen employing  $\beta$ -lactamase induction was developed by workers at Millennium [171] who discovered that a chromosomal (AmpC type)  $\beta$ -lactamase from *Citrobacter freundii* when present (with its regulator, AmpD) on a plasmid in a permeable *envA* (*lpxC*) strain of *E. coli*, was induced by inhibitors of many steps of peptidoglycan synthesis, not only  $\beta$ -lactams inhibiting transpeptidase. The relatively high throughput screen was run in microplate format and used nitrocefin as an indicator of  $\beta$ -lactamase induction. Fosfomycin, cycloserine, cefoxitin, vancomycin, moenomycin and ramoplanin were active in this screen, as was a temperature sensitive *murG* mutant at the non-permissive temperature. The permeable strain was





**Fig. 2.11** *Synergy screen*. Plates are prepared as for standard agar diffusion (Fig. 2.3). A subinhibitory concentration (generally 1/4–1/8 MIC) of cycloserine (or any antibiotic to be tested) is added to the molten agar of half the plates. Samples are applied to plates with and without cycloserine. After incubation, zone sizes on plates with and without cycloserine are compared and initial hits with a differential in zone diameter of 5 mm or greater are selected for retesting

sensitive to some membrane perturbants that gave false positives but allowed the detection of the larger, usually gram-positive specific peptidoglycan synthesis inhibitors like vancomycin and ramoplanin. It may well be that this  $\beta$ -lactamase induction screen was used for the detection of inhibitors of the cytoplasmic steps of the *mur* pathway that were disclosed by Wyeth (which had been collaborating with Millennium), without revealing the screening procedure; [53, 108]. The induction screen was also used in tandem with a secondary specific assay for inhibitors of late steps in cell wall synthesis (stages II and III; see Fig. 2.4) [36]. Inhibitors of transglycosylase and of MraY (Fig. 2.4), including muraymycin (Fig. 2.5) [119] were found in this screen.

## 2.4.9 Synergy Screens

Based on the idea that a sequential blockade of the cell wall pathway will lead to synergy of inhibitors within the pathway [42], Kuroda screened for substances whose activity was increased on a plate containing D-cycloserine (Fig. 2.11) [102]. A compound, FR-900130 (L-2-amino-3-butynoic acid) was isolated, which was a spheroplast former found to be an inhibitor of *S. aureus* alanine racemase.

In the 1990s, when the incidence of MRSA was strongly increasing, a number of groups reported on synergists of  $\beta$ -lactams (such as methicillin), against MRSA. A Microcide patent [20] described and claimed synergy screens for potentiators of antibacterial agents against strains resistant to those agents; the antibacterials included glycopeptides, macrolides, quinolones, tetracyclines, aminoglycosides and an exhaustive list of  $\beta$ -lactams. Examples were given of a number of sterols that potentiated the activity of methicillin against MRSA. Other programs did not explicitly mention screening programs, but it is highly likely that synergy screening was involved. Among synergists of methicillin against MRSA were components of tea extracts [199], carbobenzoxy-diphenyl-ala-pro-phe [45], and TritonX-100 [101].

## 2.5 Screens for New Members of Previously Described Classes of Antibiotics

### 2.5.1 Aminoglycoside Screens

Recognizing the success of screening programs using strains specifically hypersensitive to  $\beta$ -lactams and other cell wall agents, scientists at Bristol-Myers Tokyo extended the approach to aminocyclitols and aminoglycosides [130]. For the screening strain, they started with a *Klebsiella pneumoniae* isolate, Kp-8, carrying an R-factor rendering the strain highly resistant to a variety of antibiotics including gentamicin and subjected it to serial mutagenesis with NTG followed by replica-plate screening for isolates with increased sensitivity to sorbistin, an aminocyclitol antibiotic produced by a eubacterium (rather than a Streptomycete). The screening strain, Kp-126 was highly sensitized to all aminoglycosides tested (MICs  $< 0.2 \mu\text{g/ml}$  vs  $\geq 25 \mu\text{g/ml}$  on the parental Kp-8 strain for all but 4'-deoxybutirosin and amikacin which had MICs of  $3.1 \mu\text{g/ml}$ ). MICs for other classes of antibiotics were much less affected. Cephalosporin C was the most affected, its MIC decreasing 32-fold. Fermentation samples of about 20,000 soil isolates (presumably eubacteria) were screened using paired strains Kp-8 and Kp-126 in an agar diffusion assay with paper discs. Ten antibiotics showed increased activity on Kp-126. Seven previously detected compounds were detected (sorbistins, butirosins, capreomycin, BMY-28160 [a peptide antibiotic], and streptothricin); two were unidentified antibiotics of "uninteresting activity" and one was a novel amino sugar antibiotic, BMY-28521 produced by *Bacillus pumilis*, identified as 3, 3'-neotrehalosadamine (Fig. 2.12) [185].

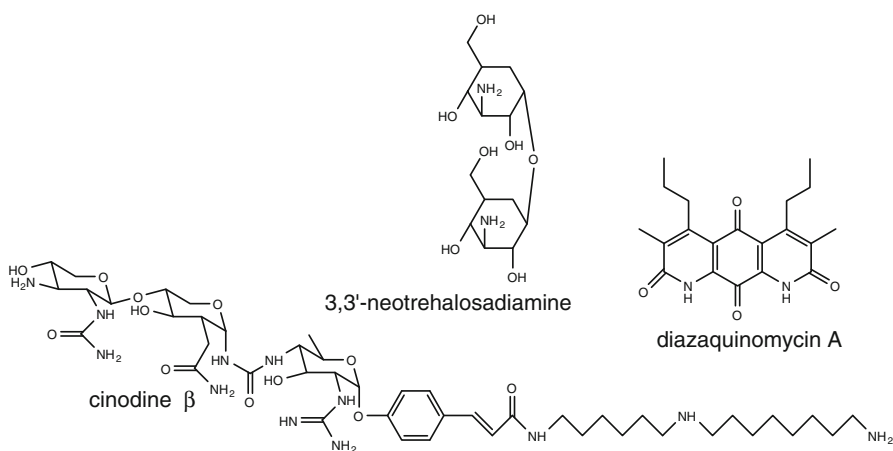


Fig. 2.12 Actives from non-cell wall directed screening

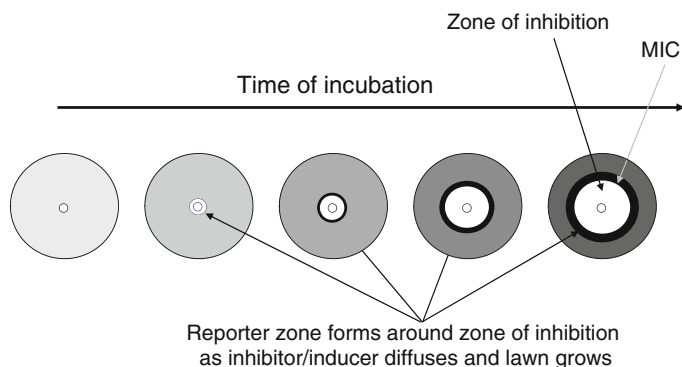
The basis for the apparent selective hypersensitivity of Kpn-126 for aminoglycosides is unknown and might certainly have led to biases in the types of compounds selected depending upon whether the differential was based on permeability, efflux, active transport, target alteration or a combination of these. However, the validity of such a directed natural product screen is based on its sensitivity combined with relatively low hit rate, reasonably low percentage of false positives and identification of novelty. There will always be false negatives in screening, but the enterprise should be pragmatic and results oriented.

A more specific and very sensitive ELISA-based screen for aminoglycosides was developed at Lilly [200]. ELISA plates were coated with anti-gentamicin polyclonal antibody and incubated with gentamicin-alkaline phosphatase conjugate. The amount of conjugate bound after washing was quantitated by measuring alkaline phosphatase activity after addition of the substrate p-nitrophenyl-phosphate. Aminoglycosides, except for neomycin B and C, competed with the conjugate for binding to the gentamicin antibody and no competition was seen with non-aminoglycosides. The ELISA assay was very sensitive, detecting gentamicin at 10 pg/ml, and insensitive to interference by substances in fermentation broths. While screening results were not shown, tests with known producers of aminoglycosides demonstrated the feasibility of the approach. The authors note that this type of methodology could also be used for the elimination of known classes in a screening cascade.

## 2.5.2 Reporter-Based Screening Platforms

A classical method is the use of indicator plates for the expression of  $\beta$ -galactosidase [121], an enzyme that can be used as a reporter of gene expression when fused to a suitable promoter. The standard “zone of inhibition” (ZOI) assays employed for empirical screening of fermentation broths and chemicals (Fig. 2.3) were adapted starting in the 1970s for use with various dyes and chromogenic or fluorogenic substrates so that reporter genes could be used to monitor the inhibition or induction of genes caused by interaction of a compound with a molecular target (Fig. 2.13). Eventually, these assays were transferred to liquid format, first in 96-well microplates and later much higher order vessels. But one beauty of the agar plate method is lost in liquid, the formation of a concentration gradient of the test compound, such that the effect of a broad range of concentrations (below and above the “minimal inhibitory concentration”) can be observed with a single sample (Fig. 2.3); this is important when a desired phenotypic readout is only seen at a specific or narrow range of concentrations, often below the MIC. Thus, on an indicator plate using samples on filter discs, a ZOI may be seen with a surrounding zone of color indicating expression of a specific enzyme (such as  $\beta$ -galactosidase) (Fig. 2.13).

The use of  $\beta$ -galactosidase, as a reporter in screening for several classes of antibiotics, was demonstrated by Kirsch and coworkers at Squibb [97]. For tetracycline, chloramphenicol, and macrolides, the reporter gene was fused to promoters of drug-specific inducible resistance promoters: tetA/tetR from Tn10 in *E. coli*, *B. subtilis*



**Fig. 2.13** Reporter screen using agar diffusion format. Reporter screens are run in the same way as standard agar diffusion screens (Fig 2.3), except that (1) the bacterial strain used contains a reporter gene ( $\beta$ -galactosidase, for example) under the transcriptional or translational control of a regulatory element that leads to turn-on of the reporter gene in response to the presence of a specific type of antibacterial inhibitor, and (2) the agar contains an indicator substrate or dye that will signal the expression of the reporter (for example, Xgal, a chromogenic substrate of  $\beta$ -galactosidase). In a strain in which  $\beta$ -galactosidase is under control of an SOS promoter, such as the *sfiA* or *recA* promoter, a DNA damaging agent or certain inhibitors of DNA replication will lead to upregulation of the  $\beta$ -galactosidase gene and a zone of blue (indicating Xgal hydrolysis) will be formed around the ZOI, at a concentration of inhibitor below the MIC

carrying the *S. aureus cat86* (chloramphenicol acetyl transferase) promoter, and *B. subtilis* carrying *S. aureus ermC* promoter, respectively. At the time this work began (although sadly not true by the time of its publication), no vancomycin resistance mechanisms were known, so a promoterless-*lacZ*-containing transposon library in *B. subtilis* was screened for strains containing promoters inducible by vancomycin. In each system, induction of  $\beta$ -galactosidase was monitored by an agar diffusion method using a specific pH indicator (2,3,5-triphenyltetrazolium chloride), chromogenic (6-bromo-2-naphthyl- $\beta$ -D-galactopyranoside) or fluorogenic (methyl-umbelliferyl- $\beta$ -D-galactoside) substrate. For each screen, suitable control constructs were used to counterscreen against non-specific inducers and artifacts. This type of screen is designed to be more sensitive than a simple ZOI readout, since the reporter expression is seen outside the ZOI, at sub-MIC levels. The tetracycline screen was eightfold more sensitive than a ZOI readout. Workers at Lederle published a similar screen for tetracyclines in the same time period [31].

The vancomycin construct was tested for inducibility by other glycopeptides which were, as hoped, shown to be active [97]. Sensitivity of the screen was not markedly better than for the ZOI readout, most likely because glycopeptides are large, generally slightly basic and tend to diffuse slowly in agar, thus limiting the size of the indicator zone. When used in screening several thousand broths, the screen yielded a number of actives that were secondarily tested for decrease in zone size in the presence of diacetyl-L-lys-D-ala-D-ala. A variety of glycopeptides were detected by the assay and no other known actives were detected, indicating the validity of the assay.

Early studies of the regulation of vancomycin resistance in *E. faecalis* led Handwerker to the interesting finding that inhibitors of transglycosylase, moenomycin as well as vancomycin, induced the VanB operon [66] whereas it was already known that teicoplanin did not induce VanB. It was later shown that the VanA and VanB operons of *E. faecium* and *E. faecalis*, respectively, are both under the control of a two component system, VanR (intracellular response regulator) and VanS (extracellular sensor) [8, 50]. A number of groups used various reporter fusions to determine what types of compounds were recognized by VanS and hence could induce these two operons, both as an academic pursuit and in order to screen for novel antibiotics. Depending upon the strain background, reporter and screening format used, varying results were obtained. While it is clear that vancomycin and teicoplanin induce VanA while vancomycin but not teicoplanin induce VanB, other results were contradictory. Ulijasz found that a variety of inhibitors of cell wall synthesis induced the VanA operon, monitored by a *lacZ* reporter fusion, when cloned into *B. subtilis* [186], while Lai found that glycopeptides, bacitracin, moenomycin and some membrane active agents induced VanA in an *E. faecium* indicator strain using a *cat* fusion [105]. Scientists at Millennium used an *E. faecalis* VanB strain with a *lacZ* reporter for screening and reported on discovery of a transglycosylase inhibitor from a fungal source, thielavin (Fig. 2.7). No breakthrough compounds came from these screens, but the methodologies presaged later screening modes.

As should be obvious, the survey for promoters induced by specific known drugs could be extended in many ways. While the Squibb screens noted in this section were designed to find new members of old classes, the screens and assays based on vancomycin resistance operons appeared, at least in some forms, to find a variety of types of inhibitor. The concept arises naturally of looking for promoters inducible by different inhibitors of several steps of a pathway, or by stress such as the SOS response discussed below (Sect. 2.6.2). Indeed, many groups recently using modern genomic era methodologies, a subject covered in a later chapter, have carried this out.

## 2.6 The Concept of Phenotypic Screening

The cell wall screens in Sect. 2.4 were generally based on phenomena caused by known inhibitors of peptidoglycan synthesis. The microbial genetic advances of the 1960s through the 1980s led to a general understanding of the essential functions of bacteria via conditional mutants and to great facility in devising selections for mutations, which would produce a desired phenotype. As the mechanism of action of the first wave of antibiotics was understood, it became evident that many of them had specific targets, and that inhibition of them might be mimicked by the effect of conditional mutations. Conversely, the phenotypes of conditional mutants when grown under non-permissive or semi-permissive conditions might be exploited for screening of inhibitors mimicking these effects. Indeed, the essential genes revealed by

microbial genetics were the potential targets for future antibacterial agents: (It should be noted that all the work of the genomics era starting in 1995, has turned up few if any targets that had been unrecognized previously.) Thus, starting in the 1970s, screens were devised that are analogous to the methods used to select for bacterial mutants, that is, by looking for the appearance of a specific phenotype that reflects the desired changes to the genotype. In drug screening, one can look for changes in appearance, behavior or biochemistry of bacteria that reflect inhibition of desired bacterial targets. Thus, screening for antibiotics took on the character of screening for mutants.

Like mutant hunts, screens could be of the “brute force” type, as might be used when screening a population for an auxotroph by replica plating. In drug screening mode, this would involve comparison of two (or more) conditions, for example, a pair of sensitive and resistant strains to find cross resistant compounds. This could be contrasted with a more selective screen, analogous to selecting for a revertant of an auxotroph to prototrophy (or a for resistance to an antibiotic), where the only colonies that appear are the desired mutants (at least in theory!). The screens of Squibb and Lederle for tetracyclines by use of *lacZ* under *tet* promoter control are the selective counterpart of a screen for differential between a sensitive and tet-resistant pair. Using two conditions as opposed to one for a primary screen is not especially “brute force,” but use of a single-condition primary screen can preserve limited sample sources.

### **2.6.1 Folate Pathway Revisited: Interplay of Genetics, Biochemistry, and Screening Strategies**

Since there are few examples in the literature of phenotypic screens, the following ramble is presented as a means of illustrating the way genetic and biochemical information can be integrated in various ways to establish phenotypic screens.

In *E. coli*, growth in minimal medium in the presence of trimethoprim, an inhibitor of dihydrofolate reductase (DHFR) at 5–10 µg/ml and thymine at 50 µg/ml leads to selection of thymidylate synthetase (*thyA*) mutants [165] because tetrahydrofolate (THF) is required catalytically for other synthetic steps (serine, methionine, purines), but THF is oxidized during synthesis of TMP by ThyA, [17]. Thus, loss of ThyA (as long as thymine or thymidine is supplied) spares THF and allows growth in the presence of a DHFR inhibitor like trimethoprim. Hence, in the presence of thymine, *thyA* mutants are more resistant to trimethoprim than *thy*<sup>+</sup> isogenic strains. It was also found that in media supplying amino acids and purines, sensitivity to trimethoprim is dependent upon thymidine concentration; under these conditions, thymidine raises the MIC of trimethoprim [100].

Taking these findings into account, Omura described a screen for natural product inhibitors of folate synthesis [136]. The screen employed *E. faecium*, which (like humans) lacks the dihydropterate synthase enzyme (FolP, target of sulfanilamide) and therefore requires exogenous pterate (or other folate). Omura reasoned that in a

medium providing amino acids, purines and a limiting amount of pterate, inhibitors of DHFR would not allow growth of *E. faecium* in the absence of added thymine but should allow growth in its presence (much as was seen by Koch in *E. coli*). Such a screen was run on 8,000 soil isolates. Four known and three unknown antibiotics were found. The known antibiotics showdomycin and oxazinomycin were reversed by other nucleosides as well as thymidine; tirandamycins A and B, known inhibitors of RNA polymerase were slightly reversed by thymidine but not other nucleosides. The novel compounds were AM-8402, an inhibitor of bacterial DHFR, and diaz-aquinomycins A and B (Fig. 2.12) which are ThyA inhibitors [122]. Indeed, detection of ThyA inhibitors would have been predicted based on the simple analogy to a mutant screen for a thymine auxotroph.

Based on the folate system, one might envision other screens: (1) a screen for DHFR inhibitors using a pair of *thyA* + and *thyA* isogenic *E. coli* strains grown in the presence of thymine; the *thyA* strain should be more resistant to DHFR inhibitors but that resistance would be function specific, not compound specific. (2) A selective screen for ThyA inhibitors, quite analogous to the original selection for *thyA* mutants. A *thy* + strain of *E. coli* would be used to inoculate a plate containing 5 µg/ml trimethoprim (the amount of trimethoprim would be selected to just prevent growth of a lawn after incubation) and 50 µg/ml of thymine. Samples would be applied on discs or in wells and overnight incubation allowed. As thymine is present, ThyA inhibitors should not give a ZOI but instead should promote growth in the presence of trimethoprim (i.e., give a zone of exhibition (ZOE)). This screen (and all antibacterial screens, for that matter), must be piloted with a large set of known antibiotics and, if natural products are to be tested, with a large number of fermentation samples to ascertain that there is a very low hit rate and that the screen is not intrinsically sensitive to common false positives. In this case, there might be common medium components or other compounds that promote growth of the lawn for other, unforeseen reasons. As a rule of thumb, the hit rate for natural products should be <0.1% since a higher hit rate would not be expected for an inhibitor of a specific enzyme for which natural product inhibitors are rare or unknown.

### 2.6.2 Phenotypic Screening for DNA Replication Inhibitors

When the fluoroquinolones ciprofloxacin and norfloxacin, which were known to target DNA gyrase, entered clinical trials in the early 1980s, it was quickly realized that other DNA replication enzymes were potential antibacterial targets as well. (DNA topoisomerase IV was not recognized as a second target of the fluoroquinolones until 1994 [95, 96].) In fact, this author began her career in antibacterial discovery in 1982 based on her academic research interests in bacterial DNA replication. Although, as noted repeatedly here, the ongoing screening programs were not published upon until much later or not at all, it should be stated that phenotypic screening for inhibitors of DNA gyrase as well as other inhibitors of DNA replication was ongoing during the 1980s and beyond. A 1990 review proposed DNA replication



proteins as suitable antibacterial targets, but mentioned no screens [156]. As most of these screens are not in the public domain, the academic work that was ongoing and which provided fodder for development of one type of DNA screen will be reviewed here briefly, as will some of the eventually published screens.

The SOS hypothesis first enunciated by Evelyn Witkin in the mid 1970s [193, 194] proposed that UV-induced mutations are produced by an error-prone repair system whose induction is coordinated with other *lexA*+/*recA*+ dependent processes, such as prophage  $\lambda$  induction and filamentous growth, in response to UV irradiation, thymine starvation, DNA damaging agents such as mitomycin C or other inhibitors of DNA synthesis such as nalidixic acid. Schuster had already shown that prophage  $\lambda$  is induced under non-permissive conditions by strains carrying temperature sensitive mutations in the DNA replication genes *dnaB*, *dnaE*, and *dnaG* but not *dnaA* [151]. Taken together, this implies that screens devised to monitor SOS induction should detect DNA damaging agents and DNA synthesis inhibitors.

Colorimetric tests for inducers of prophage  $\lambda$  were described that could be used to screen chemicals and natural products for DNA damaging agents acting as carcinogens or with potential use as anticancer agents [46, 47]. These screens employed a permeable *envA* (= *lpxC*) *E. coli* strain containing a  $\lambda$ -*lacZ* fusion prophage in which induction could be monitored by measurement of  $\beta$ -galactosidase. In 1982, a reporter strain (the SOS chromotest strain) for inducers of the SOS response that used the *lacZ* gene fused to the *sfiA* (= *sula*) promoter was described [145]. Thus by the mid 1980s, it was clear to industrial scientists involved in antibacterial screening that such indicator strains, which were readily available at the time, should respond to fluoroquinolones (as they were the descendants of nalidixic acid, a known inhibitor of DNA gyrase [58]) and inhibitors of a subset of DNA replication functions. It was later shown directly, through use of a strain in which the *recA* promoter was fused to *lacZ*, that fluoroquinolones did, indeed, induce the SOS response [144]. Of course, such strains also responded to DNA damaging agents of certain types. So any antibacterial screen using such strains would have had to be used in conjunction with counter screens against frank DNA damaging agents and secondary screens for specific inhibition of DNA replication proteins and/or DNA gyrase.

The first published use of an SOS-type screen for gyrase inhibitors is that of Osburne [138] using *B. subtilis* (since it is much more permeable than *E. coli*) with *lacZ* under control of a damage-inducible *din23* promoter. Actually, two strains were used, one containing in addition to the *lacZ*-*din23* fusion, a *recM13* mutation and the other a *recM*+ allele. As certain DNA damaging agents induced *lacZ* expression in either the *recM*+ or *recM13* backgrounds, but true DNA gyrase inhibitors (nalidixic acid, norfloxacin and novobiocin) induced *lacZ* on both strains, compounds that induced in both strains were selected as hits. Cinodine, a natural product inhibitor of DNA gyrase (Fig. 2.12), was identified by the screen.

Another illustration of the application of academic reports of interesting phenotypes of mutants in potential antibacterial target genes is a screen for inhibitors of Gyrase B supercoiling [65]. The screen relied upon the growth-dependence of a Topoisomerase I (*topA*) deletion mutant of *E. coli* upon inhibitors of supercoiling. This was based on the initial observations of DiNardo [39] that, in *E. coli*, *topA*



deletions selected rapidly for compensatory mutations in *gyrB* or *gyrA* and of Stankiewicz [166] that an amber mutant of *topA* in a *ts*-amber suppressor background was dependent upon novobiocin for growth. Thus, the screen [65] used a strain of *E. coli* transduced with a *topA* deletion that required novobiocin, or another suitable inhibitor, for rapid growth at 28°. When plated on agar in the absence of such a compensating inhibitor, the strain gave a very pale lawn. However, when filter discs containing *gyrB* inhibitors were placed on the plate before incubation, luxuriant growth was seen after incubation in the area of the plate where the compound had diffused from the filter, giving a zone of exhibition (around a zone of inhibition). No results of the assay were disclosed, but novobiocin and coumermycin were used as positive controls, and could be found upon screening.

## 2.7 Why Rational Screening Has Had a Low Yield: Implications for Current Programs

Aside from the successes in the cell wall area (the discoveries of the carbapenems, monobactams,  $\beta$ -lactamase inhibitors, fosfomycin, ramoplanin, new glycopeptides) and despite programs designed to find new antibacterial classes by target-directed methods, rational screening for antibacterials has been remarkably unsuccessful; this applies not only to the largely shrouded whole-cell phenotypic screens of the 1970s through 1990s, but to the more openly described genomics-based and biochemical screens that have followed. Several factors could be implicated for this low output, but a major problem has been the mismatch between the sample sources used and the screening strategies.

The early mechanism-based screens (at least those that were disclosed!) were mostly cell wall directed. They were used to screen natural product sources and served in part as dereplication tools. The concept, born in the early chemotherapeutic era, of choosing candidate targets for their likelihood of selectivity is valid and deserves weight. The choice of the bacterial-specific peptidoglycan synthesis pathway over, say, membrane lytic or DNA damage as mechanisms to be exploited for directed antibacterial screening is logical. But inhibition of cell wall synthesis may have been a singularly rich source of morphological and phenotypic screening possibilities – where inhibition of any point in a long pathway could give rise to similar phenotypes or phenomena. With the revelation by microbial genetics and later genomics, of essential bacterial genes that could be considered targets for new antibacterials, screening became directed more and more toward specific single targets. But the narrowing of target choice was counterproductive for natural product screening. Such highly rational (and hence politically appealing) targeted screens competed successfully with broader pathway (and other) screens for limited screening slots in natural product programs. The beauty of the natural products source is the likelihood that these compounds have evolved, at least in part, to inhibit growth of bacteria [37] (although this has been argued against as the main *raison d'être* of microbial secondary metabolites [109, 201]), and while the molecular targets (or, let

us say, receptors) are unknown, they are preselected. If a natural product inhibitor has not already been found for a newly selected target, it is likely that such an inhibitor is rare, since all essential targets have been screened for empirically for decades. To make the most of natural products requires screens that can detect the widest breadth of chemical novelty.

The lure of target-based screening was a reasonable development from the chemotherapeutic concepts developed in early anti-infective and cancer therapies. Certainly, in the 1980s, success in cardiac pharmacology, with angiotensin converting enzyme (ACE) inhibitors and the HMG Co-A reductase inhibiting statins, laid the groundwork for such screens. Furthermore, the idea that chemical inhibitors could be designed for enzyme targets was also rational. Indeed, for chemical collections, target-based screening made theoretical and logical sense. The innate problems of natural product screening and the need to dereplicate before chemical isolation is obviated when the structure of the hit is known. However, the quality of chemical libraries used for antibacterial screening during the 1980s and even now, has been poor, in the sense that most antibacterial hits are surfactants or otherwise generally toxic and, for some reason, it is hard to find hits even for biochemically screened enzyme targets [142]. It is likely that the nature of the libraries has been skewed toward non-anti-bacterial structures. But a critical problem is that bacterial entry, especially into the gram-negative cytoplasm, requires characteristics not prevalent in standard libraries [131, 142]. Gram-negative entry favors polar, charged molecules for outer membrane entry and an uncharged species for cytoplasmic membrane passage [131, 160]. Along with the mismatch of screens and sources, the concentration on preselected targets has led naturally to a focus on single enzyme targets and this emphasis leads to a high potential for rapid resistance development [157, 159]

Among natural products, there has been a long-standing push for use of organisms from novel niches, and more recently, for culturing or cloning unculturable organisms [12, 86]. Furthermore, screens of natural product sources should be hypersensitive (exploiting the likelihood that previous searches might have missed compounds in low concentration) and, above all, aimed at finding novelty.

How can this be done? As later chapters will note, a variety of whole cell screening modes using under-expression of specific genes have been used. For efficient use in natural product screening, these should be deployed in groups, in banks of screens. In this way, a specific hit in a single screening organism (among others in a bank) would indicate selectivity, and likely novelty. These banks should be used in primary screening, to make use of the sensitivity of under-expression. For chemical screens, specific phenotypic assays should be used either as primary screens or to immediately validate that an *in vitro* hit with antibacterial activity actually targets the desired enzyme. In many, if not most, cases, the antibacterial activity of a hit in an enzyme assay is due to non-specific, often surfactant activity (especially if selection is for anti-gram positive activity). Similarly, optimization of inhibitors via medicinal chemistry is often found to make a quantum leap from lack of antibacterial activity to possession of such activity. It is critical to monitor such optimization with a specific phenotypic screen or assay to avoid chasing non-specific effects.

Correlation of antibacterial activity and anti-enzyme activity is insufficient to support the assumption of causality. While it is perfectly reasonable to develop an antibacterial with multiple modes of action, it is more likely that toxicity will be a problem if the “second” mode is non-specific, surfactant, and alkylating, or otherwise toxic. Thus whole cell phenotypic assays that tie antibacterial activity to action against a desired cellular target are still highly desirable. They may have failed to provide us with novel antibacterial classes over the last 30 years, but there are ways in which such phenotypic screens can be better designed and deployed against improved sample sources to find new entries into the rapidly obsolescing antibacterial armamentarium.

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