Chapter 7

TARGET DIRECTED ANTIMICROBIAL SCREENS

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Introduction

During the so-called "Golden Era" of antibiotic research, from the mid-1940s to the mid-1960s, the rate of discovery of new microbial metabolites did not significantly change [Bérdy, 1974]. Subsequently it accelerated slightly, and there was a dramatic increase between 1972 and 1978, during which period the discovery rate jumped from 180 to 340 new antibiotics per year [Perlman, 1977]. The fact that it has become increasingly difficult to find antibiotics which are both novel and useful is evidenced by the drop in the clinical success rate of from 5% before 1960 to less than 1% today.

In the early days, many millions of different microorganisms, mainly actinomycetes isolated from soil, were screened by the classical methods of Waksman and others. Woodruff and MacDaniel [1958] reported that the screening of 10,000 microorganisms resulted in the isolation of one clinically effective agent. Subsequently, Nelson [1961] described a screening programme involving 400,000 cultures over a 10 year period which led to the discovery of three utilizable drugs. More recently, the technique and results of a 25 year screening programme were appraised by Woodruff and others [1979]. Thus, out of 21,830 isolates examined in one year 2 were selected as having therapeutic potential.

One of the problems associated with traditional forms of screening has been the early elimination of cultures producing known antibiotics; most having relied on the comparative antimicrobial spectrum using a suitable set of antibiotic-sensitive and -resistant organisms. By a combination of agar diffusion and thin layer bioautograms it is usually possible to eliminate the majority of cultures, and the survivors are then progressed for extrac-

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tion and chemical characterization of the antibiotic. However, the problem of identification has escalated markedly as the number of antibiotic structures, often poorly described, has increased year by year. In the mid-1950s approximately 300 antibiotics were recognized [Bérdy, 1974] whereas today there are probably 10 times as many from actinomycetes alone. This has led to computerization of physico-chemical and biological properties, and suitable identification programmes have been developed [Bostian *et al.*, 1977]. Further improvements in efficiency have been obtained by developing more selective screening strategies.

Selective Search Strategies

Selective search strategies can be divided into three main types. Target directed screening either focuses on the target site for known antibiotics, where congeners or alternative structures are sought, or it uses rationally-selected enzyme or receptor sites for which there are no known synthetic or naturally occurring inhibitors. The second type of selective search involves the use of unusual biological or chemical test procedures to select for rare types of activity or to detect particular moieties in the chemical structure of metabolites. A third type of selective approach could be the detailed examination of new species or relatively unexploited groups of microorganisms. For the sake of simplicity we shall consider these approaches under the two broad headings of "target directed screens" and "other selective search methods" (Table 1).

TABLE 1

Selective search strategies

(i) Target Directed Screens
Use of antibiotic resistant assay organisms.
Use of antibiotic supersensitive assay organisms.
Selection of antibiotic producing isolates for resistance to required antibiotic types.
Inhibition of antibiotic inactivating enzymes.
Inhibition of target enzymes or receptor site.
(ii) Other Selective Screen Methods
Induction of morphological aberrations.
Inhibition of differentiation.
Detection of chemical moieties.
Antimetabolite screens.
Examination of rare organisms.
Potentiator screens.

Target Directed Screens

Screens based on resistance and sensitivity to known antibiotics Comparison of culture broth inhibition of parental strains of bacteria with activity against isogenic progeny made resistant to particular antibiotics can be used to detect cultures producing similar types of antibiotics. This approach can be modified by incorporating antibiotics in the isolation medium to pre-select isolates capable of producing analogues of known antibiotics. For example, strains of actinomycetes which produce aminoglycoside antibiotics are known to produce phosphotransferases and acetyltransferases which selectively inactivate these structures [Dowding and Davies, 1975]. The majority of actinomycetes are very sensitive to aminoglycosides and thus the inclusion of these antibiotics in the isolation medium could be used to select for putative producers of known and alternative aminoglycosides. This is potentially more valuable if the mechanism of resistance in the producing organism involves a modified target site, as in the resistance of *Streptomyces azureus* to thiostrepton [Cundliffe, 1978].

Mutants supersensitive to known antibiotics can lead to the selection of cultures producing analogues of a particular class of antibiotic. Recently, this approach has been successful in the search for new β-lactam antibiotics [Aoki *et al.*, 1977; Kitano *et al.*, 1977].

Inhibition of antibiotic inactivating enzymes Where clinically important antibiotics have met with increasing difficulties due to resistance mechanisms involving antibiotic inactivation, this may be overcome either by developing more stable analogues or by adding a second compound to inhibit the inactivating enzyme. The latter approach has been applied to penicillin antibiotics and has led to the discovery of β-lactamase inhibitors such as clavulanic acid and the olivanic acid complex. This aspect is covered in more detail in the chapter by Dr Hood.

Specific target-site-directed screens These screens may utilize either cell-free enzyme reactions or whole cell systems. Although the former approach has been optimized for pharmacological targets and has yielded a number of metabolites with clinical potential (see Hamill, this volume) there have been surprisingly few reports on its application to antimicrobial screens [Hamill, 1977].

The most popular target-directed approach has been to follow incorporation of specific radiolabelled substrates into the macromolecules of intact cells. Omura *et al.* [1979] reported a two-stage screen for cell wall inhibitors using as a primary screen activity against *Bacillus subtilis* in the absence of inhibition of *Acholeplasma laidlawii*. Positive cultures were then tested for

inhibition of incorporation of *meso*-diaminopimelic acid into *Bacillus* sp. ATCC 21206 (Dpm^-). This resulted in the detection of 6 known antibiotics and one new type, the azureomycins, from 10,000 soil isolates. This approach is subject to pleiotropic effects of inhibitors, active at one stage of biosynthesis, on earlier steps. The azureomycins, for example, rather than specifically inhibiting incorporation of Dpm into the growing pentapeptide, lead to the accumulation of UDP-Mur-*N*Ac-L-Ala-D-Glu-*meso*-Dpm-D-Ala-D-Ala [Spiri-Nakagawa, 1979]. Thus their site of action is probably inhibition of formation of the undecaprenyl-disaccharide-pentapeptide cell wall precursor.

A similar screen based on incorporation of radiolabelled *N*-acetyl glucosamine led to the discovery of gardimycin. Again, this inhibitor seems to prevent transport of the disaccharide pentapeptide rather than to inhibit any of the alternative stages of peptidoglycan biosynthesis or cross-linking [Somma *et al.*, 1977].

The alternative approach of using *in vitro* enzyme reaction assays to screen for antimicrobials has received scant attention to date. A number of systems mentioned in the literature tend to be retrospective, describing the precise mode of action of an antibiotic discovered by another technique. Thus, both polyoxins and nikkomycins have been shown to inhibit chitin synthase [Gooday and De Rousset-Hall, 1976] but no inhibitors have yet been discovered by using the free enzyme as a test. The advantages and problems associated with specific enzyme screens will be described later using as an example a system that we have developed to detect β-lactam antibiotics.

Other Selective Screen Strategies

Assay techniques which test for induction of morphological aberrations or the inhibition of a specific process of differentiation have led to the discovery of numerous antibiotics. Thus, nikkomycins inhibit zygospore production in *Mucor hiemalis* [see Zähner, this volume].

A wide variety of chemical reagents have been applied to t.l.c. plates to detect particular chemical moieties. These include Dragendorff (tertiary amine), Ehrlich (indole), Sakaguchi and Wood (guanidine) and tetrazolium chloride (reducing substance reagents) which have resulted in the discovery of a range of metabolites including dienomycin [Umezawa *et al.*, 1970] and KD16-U1 [Tätsuta *et al.*, 1974].

The rationale for, and the success of, antimetabolite screens have been well reviewed by Pruess and Scannell [1974] and although the value of compounds detected in this manner has been questioned from time to time, increasingly this approach is being evaluated in the search for new antitumour agents.

The testing of culture broths as potentiators of known antimicrobial agents should be distinguished from screens for inhibitors of antibiotic inactivating enzymes. Antibiotic potentiation or synergy has been demonstrated for a number of antibiotic combinations including mecillinam and other β-lactam antibiotics [Wise *et al.*, 1976] and more recently for D-cycloserine or β-lactam plus the synthetic antibacterial agent alaphosphin [Allen *et al.*, 1978]. Kuroda and others [1980] have described the screening and isolation of a new D-cycloserine potentiator from *Streptomyces catinulae* which reputedly inhibits L-alanine racemase.

Organisms with the known ability to produce unusual secondary metabolites may repay further more detailed study. The rare species *Streptomyces clavigulerus*, one of the first shown to produce cephamycin C, was subsequently found to produce at least 14 other antibiotics. These included four novel metabolites identified as clavulanic acid [Brown *et al.*, 1976] and three related structures with antifungal activity [Napier *et al.*, 1977]. Similarly the examination of some rarer actinomycetes led to the discovery of the gentamicin C complex from *Micromonospora* species along with other potentially valuable metabolites [Nara *et al.*, 1977].

Eubacteria have often been regarded as unrewarding since their metabolites, largely isolated from *Bacillus* and *Pseudomonas* spp., have tended to be peptides and other toxic metabolites with little therapeutic value. It is possible to isolate other bacteria which apparently produce more interesting metabolites such as the chloramphenicol analogues from *Corynebacterium hydrocarboclastus* [Suzuki *et al.*, 1972]. However, unusual strains of *Bacillus* and *Pseudomonas* species were found to produce the novel aminoglycosides butirosins [Woo *et al.*, 1970] and sorbistins [Tsukiura *et al.*, 1976] the former of which led to the development of amikacin [Kawaguchi *et al.*, 1972].

In assessing the potential of the target-site-directed approach it is interesting to examine the selective action and human toxicity of currently used antibiotics.

Lethal Inhibition of a Target-site Enzyme

Increasingly, biochemists and molecular biologists have used antibiotics to aid the study of biological reactions at the enzymic and molecular level. The modes of action of virtually all clinically used antibiotics are known (Table 2) as are those of many veterinary and agricultural antibiotics.

Mode of Action of Clinically Useful Antibiotics

The majority of antibacterial drugs on the market inhibit the biosynthesis of cell walls or proteins.

TABLE 2

Mode of action of clinically-used antibiotics

Antibiotic	Target Site	Toxicity/Side effects
I. ANTIBACTERIAL		
(a) Cell Wall		
Phosphonomycin	Phosphoenol pyruvate transferase	G.I. upsets.
D-cycloserine	L-alanyl racemase D-alanyl-D-alanyl ligase	None although some central nervous system disturbances occur at very high dosage.
Bacitracin	Isoprenol pyrophosphatase	Nephrotoxic
Vancomycin	Transfer of peptidoglycan to the cell wall acceptor	Ototoxic
β-lactams	Penicillin binding proteins inc. transpeptidase and carboxypeptidase	Sensitization, rashes.
(b) Membrane		
Gramicidin) Polymixin)	Binding to the cytoplasmic membrane	) Highly toxic)
(c) DNA		
Rifamycins	DNA-dependent RNA polymerase	Rashes, G.I. upsets.
(d) Protein		
Streptomycin (Aminoglycosides)	P10 protein on the 30S ribosomal sub-unit	Eighth nerve, ototoxicity and/or renal toxicity depending on structure. Varies for different analogues. Potentiate neuromuscular blocking drugs.

Tetracyclines	Inhibits amino-acyl tRNA binding to the A site on 70S ribosomes	Gastro-intestinal upsets, slight renal and hepatic toxicity. Contra-indicated in pregnancy and children.
Chloramphenicol	Inhibition of peptide bond formation	Gastro-intestinal upsets, bone marrow depressant.
Erythromycin	Translocation of peptidyl-tRNA.	None reported.
Lincomycin	As for chloramphenicol	None. Diarrhoea in 10%. Pseudomembranous colitis.
Fusidic acid	Translocation of peptidyl-tRNA. Inhibits EF-G dependant GTP hydrolysis	None reported.
Other		
Novobiocin	Complexes Mg^{++}, interferes with membrane function and DNA gyrase	Eosinophilia, rashes, skin eruptions.
II. ANTIFUNGAL		
Nystatin Amphotericin B (Polyenes)	Sterol complex formation resulting in membrane leakage	Highly toxic, nausea, vomiting, thrombophlebitis, anaemia, hypokalaemia, rise in blood urea.
Griseofulvin	Microtubular protein aggregation. Inhibits spindle formation during mitosis	None at dosage used.
Pecilocin	Not reported	Topical use only.

While the former macromolecular structure is unique to bacteria, and inhibitors of the early and late stages in its formation seem relatively free of toxicity, those that inhibit the transfer of the disaccharide pentapeptide to the cell wall acceptor have toxic side effects. This may be due to the existence of polyprenol-linked systems in eukaryotes. Similarly, some protein synthesis inhibitors are selectively toxic for prokaryotic translation whereas others such as fusidic acid and tetracyclines act on both 70S and 80S ribosomes. The utility of the latter type of antibiotic depends on their enhanced accumulation into bacteria compared with human host cells. Some apparently selectively toxic antibiotics, such as the aminoglycosides and chloramphenicol, have significant side effects, whereas others such as erythromycin and lincomycin have none. Fortunately the therapeutic index is sufficiently high for the former group also to be of clinical value.

Thus, a target-directed screen suffers from some degree of uncertainty as to the potential therapeutic value of the screen products since it is impossible to predict either side effects or penetration into the whole infective microorganism. However, one is less likely to detect compounds with irreversible human toxicity or with an inadequate therapeutic index.

The frequency of discovery of new substances in defined mode-of-action screens may be lessened rather than increased. However, when a new antibiotic is discovered by the selective approach, the *in vivo* success rate should be higher than in a random screen. It is a positive selection method, rather than a negative rejection approach, that can detect down to pgm amounts of compound in complex fermentation broths.

Choice of Antimicrobial Target

Many factors influence the choice of a particular enzyme target, some of which are set out later. However, two primary considerations are selective toxicity and lethality of action. Thus, the step should be peculiar to the group of problem microorganisms being considered and it should be essential for normal growth and/or infection. Three major areas for exploitation are cell wall, protein and nucleic acid biosynthesis.

Cell wall biosynthesis A number of antibiotics inhibit cell wall biosynthesis (Fig. 1) and many of these have found clinical application (Table 2). Many other known antibiotics whose mode of action has yet to be described may be inhibitors of cell wall biosynthesis. For instance, as interest in the role of polyprenol carrier systems has increased, the antibiotics amphomycin and showdomycin have been shown to inhibit enzymes involved in these reactions.

Considering the biosynthesis of peptidoglycan in

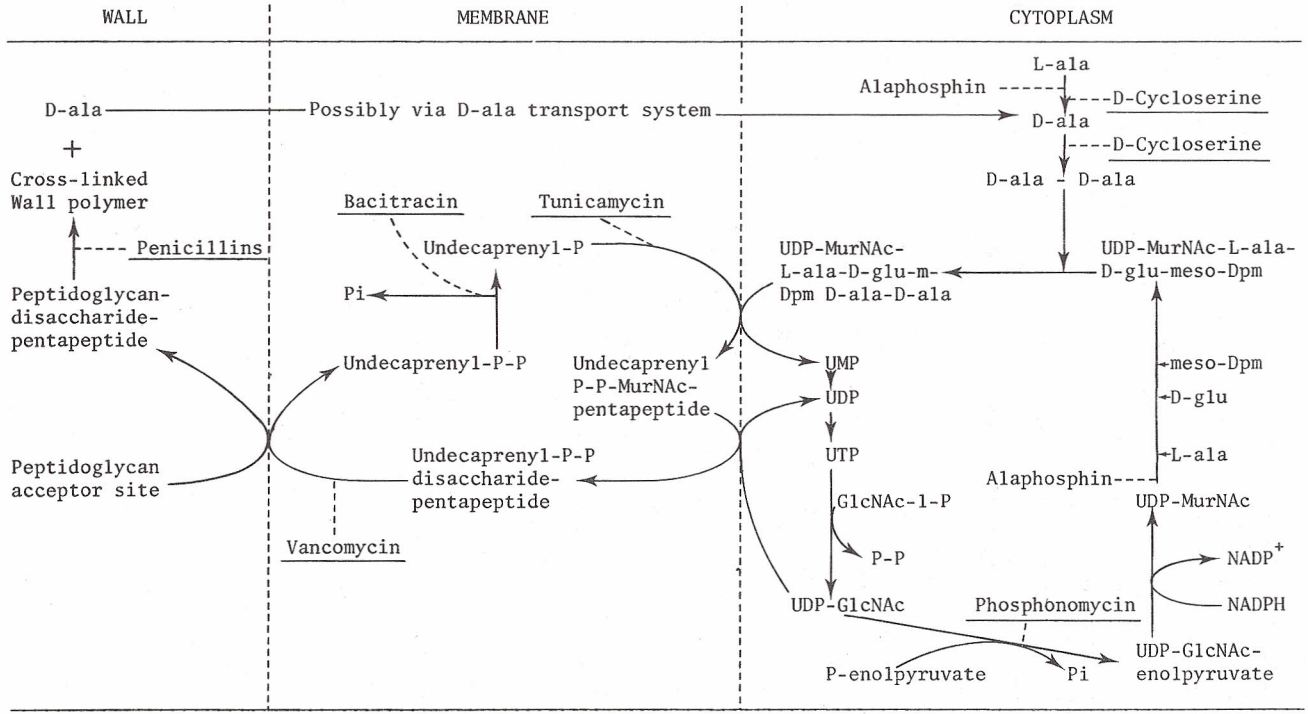

Fig. 1 Peptidoglycan synthesis and the sites of action of several antibiotics.

gram-negative microorganisms (Fig. 1) there are a number of steps which are inhibited by one or more antibiotics, and some for which there are no known inhibitors. For example, the synthetic antibacterial alaphosphin is the only agent known to inhibit the addition of L-alanine to UDP-*N* acetyl muramic acid. Techniques are now available to screen for inhibitors of the unexploited targets, with the possible exception of the attachment of the disaccharide pentapeptide to the cell wall acceptor [Omura *et al.*, 1979]. Any inhibitor would, by virtue of its lethal site of action, be a prime candidate for chemical modification to enhance enzyme affinity, cell permeability or pharmacokinetics, whichever was the limiting factor in its potential utility.

If analogues of antibiotics with a known site of action were desired, then screens could be set up for the appropriate reaction and these would be followed by secondary screens to resolve known and new compounds.

The test system can use either supersensitive mutants or the isolated enzyme. The former system has been used by Kitano *et al.* [1977], who described the use of *Pseudomonas aeruginosa* strains made highly sensitive to ß-lactam antibiotics but retaining their natural resistance to other antibiotics. A number of modified cephalosporins were found. Similar use of *E. coli* strains by Aoki *et al.* [1977] led to the discovery and isolation of the nocardicins. Our own experience in screening for new ß-lactams using a DD-carboxypeptidase preparation from *Actinomadura* R39 provides a useful illustration of the isolated enzyme approach.

Protein biosynthesis The inhibition of protein biosynthesis and the various stages which might be used to screen for new antibiotics could occupy a text book [Vasquez, 1979]. A very wide range of antibiotics inhibit protein biosynthesis and the primary sites of action are listed in Table 3. Assays are now available

TABLE 3

Inhibition of protein biosynthesis (Based on Vasquez, 1979)

Site of inhibition	Inhibitors	System Inhibited	
		Prokaryote	Eukaryote
1. Chain Initiation			
1.1. Formation and attachment of 1F-2-Met-$_t$RNA$_F$-GTP	Showdomycin	+	+
	Kasugamycin	+	?
	Aurin-tricarboxylic acid (A-TCA)	+	+
	Polydextran(PD) SO_4 Polyvinyl(PV) SO_4	+	+

TABLE 3 (Cont'd)

1.2. mRNA recognition	Edeine A1	+	+
	7-Me-GMP	+	+
	A-TCA, $PDSO_4$, $PVSO_4$	+	+
1.3. Sub-unit joining	Fluoride salts	?	+
	MDMP (Methyldinitro-arabino-*N*-Methyl-propionamide)	-	+
1.4. Positioning of initiator on the donor site	Pactamycin	+	+
2. Peptide Chain Elongation			
2.1. Aminoacyl-tRNA recognition	Tetracyclines	+	+
	Thiostrepton group	+	-
	Micrococcins	+	-
	Fusidic acid	+	+
	Lectins e.g. Ricin	(+)	(+)
	Chartreusin	+	+
	Pleuromutilin	+	-
	Aminoglycosides	+	-
	Hygromycin B	+	+
2.2. Peptide bond formation	Puromycin	+	+
	Chloramphenicol	+	-
	Lincomycin group	+	-
	Streptogramin A group	+	-
	Macrolides-Carbomycins	+	-
	Spiromycins	+	-
	Althiomycin	+	-
	Amicetin	+	+
	Blasticidin S	+	+
	Actinobolin	+	+
2.3. Translocation	Cycloheximide	-	+
	Viomycin	+	-
	Hygromycin B	+	+
	Streptogramin B	+	-
	Macrolides-Erythromycin	+	-
	Methymycin	+	-
	Lancamycin	+	-
	Bottromycin	+	-
	Spectinomycin	+	-
	Fusidic acid	+	+
3. Peptide Chain Termination			
3.1. Recognition and binding of release factors	Inhibitors of amino-acyl-$_t$RNA binding	See 2.1.	
3.2. Peptidyl-$_t$RNA hydrolase	Inhibitors of peptide bond formation	See 2.2.	
	Negamycin	+	-
3.3. Release of $_t$RNA	Inhibitors of EFG and GTP function	See 2.3.	

for most of the stages in protein biosynthesis, and it is possible to screen both for new antibiotics and for analogues of existing ones. In particular, note that there are relatively few inhibitors of the initiation reactions. Moreover, the utility of many protein synthesis inhibitors may be restricted by lack of penetration into a variety of bacteria (e.g. osteogricins) and it may be worthwhile to screen for congeners which have improved transport properties.

Nucleic acid biosynthesis Antibiotics such as azaserine and diazo-norleucine which inhibit nucleotide biosynthesis (Table 4) inhibit the same enzymes in eukaryotic systems and are toxic in man. They are not likely to be useful targets unless one can identify an enzyme for which the kinetic, structural or inhibition data suggest a sufficiently wide difference between pathogen and host. Thus, the synthetic antimicrobials of the sulphonamide or trimethoprim type are selective inhibitors of bacterial tetrahydrofolate biosynthesis.

The majority of antibiotics which inhibit nucleic acid biosynthesis tend to be non-selective but despite their toxicity they have found use in cancer chemotherapy. These antibiotics tend to intercalate between base pairs (adriamycin), form cross-links (mitomycin), bind in the minor groove of DNA (actinomycin D) or cleave DNA at GpT and GpC sequences (bleomycin).

Two enzyme targets seem to offer selectivity for prokaryotic systems. Inhibitors of DNA-dependent RNA polymerase are bactericidal and one, rifampicin, has found clinical use particularly in combination with other antitubercular drugs. Permeability problems and lack of oral absorption have to some extent restricted the use of these antibiotics. An alternative possible target is DNA gyrase, which is inhibited by, *inter alia*, the antibiotic novobiocin. Novobiocin was once used as an anti-staphylococcal agent but high serum binding, hepatic disfunction and skin reactions have curtailed its use. It is difficult to ascertain whether its host toxicity and site of action are due to its ability to complex Mg^{++} rather than high affinity for DNA gyrase [Sugino *et al.*, 1978].

A Screen for β-Lactam Antibiotics Using a Penicillin Sensitive Enzyme

Antibiotics of the β-lactam family interfere with the final steps of peptidoglycan biosynthesis. The biosynthesis of this polymer can be divided into three stages:

(1) the intra-cellular synthesis of the activated precursors UDP-*N*-acetylglucosamine and UDP-Mur*N*ac-pentapeptide;

TABLE 4

Antibiotics affecting DNA and RNA biosynthesis

Mode of Action	Antibiotics
Intercalation between adjacent base pairs	Adriamycin (1) (Actinomycin D) (2)
Hydrogen bond formation with guanine in the minor groove of DNA	Actinomycin D (2) Aureolic acid group (3)
Formation of cross-links between double stranded DNA	Mitomycin (4) Porfiromycin (4)
Single stranded scission at GpT and GpC sequences	Bleomycin (5) Phleomycin (5)
DNA gyrase	Coumermycin (6) Novobiocin (Nalidixic acid - synth)
Binding to the β-sub-unit of DNA-dependant RNA polymerase	Rifamycins (7) Streptovaricins Streptolydigin
Nucleotide biosynthesis	Azaserine (8) Diazo-norleucine Hadacidin Mycophenolic acid Psicofuranine

References

(1) Remers, W.A. [1979*a*]
(2) Remers, W.A. [1979*b*]
(3) Remers, W.A. [1979*c*]
(4) Remers, W.A. [1979*d*]
(5) Remers, W.A. [1979*e*]
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(8) Franklin, T.J. and Snow, G.A. [1975*b*]

(2) the transfer of these precursors to a cytoplasmic membrane-bound C_{55} isoprenoid alcohol phosphate. A lipid soluble intermediate is formed and, if present in the final structure, the cross bridging amino acids are added to this intermediate at the ω-amino acid of the third residue of the peptide;
(3) the final stage consists of the polymerization of the monomers outside the cytoplasmic membrane by enzymes that are themselves attached to the plasma membrane.

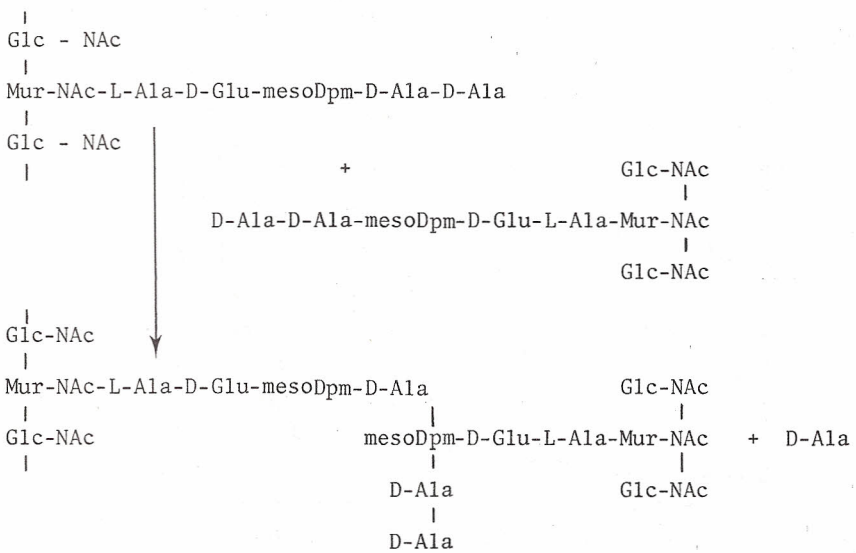
The energy of the phosphodiester bond between pyrophosphate and muramic acid is utilized for glycan chain elongation, whereas the peptide cross links are closed by a transpeptidation reaction, in which the carboxyl group of the penultimate D-ala residue of one pentapeptide unit is transferred onto the free amino group of another peptide unit (Fig. 2). Formation of the peptide bond is accompanied by the release of the ultimate D-ala residue. In the presence of penicillin the transpeptidation reaction is inhibited, causing a profound disorganization of the cell envelope of the growing cells. There are at least two major β-lactam-sensitive activities that use cell wall precursors as substrates. In addition to the transpeptidation reaction, the terminal D-ala can be transferred using water as the acceptor and this is referred to as the DD-carboxypeptidase reaction (Fig. 2). A DD-carboxypeptidase penicillin-sensitive activity has been shown to occur in many bacterial species [for references see Frére, 1977] and can be assayed by using the cell wall precursor UDP-Mur*N*ac-L-ala-D-glu-Dap-D-ala-D-ala as substrate.

Development of a high throughput assay for DD-*carboxypeptidase* In developing a cell free enzyme system to detect β-lactams a number of general criteria needed to be considered. Thus the assay should be:

- simple, with direct measurement of product rather than a coupled reaction,
- capable of automation,
- selective and insensitive to non-specific inhibition by broth constituents such as metal ions, oxidizing or reducing agents, chelating agents, pH, foreign substrates or products, enzymes. Streptomycetes, for example, produce extracellular proteases, ATPases, β-lactamases etc.,
- highly sensitive, i.e. the substrate concentration should be as low as practicable and the enzyme concentration sufficient to achieve 50% conversion to product in a short time interval. A short incubation time ensures measurement of initial reaction rates and reduces non-specific inhibition effects,
- adaptable to secondary screening of TLC or HPLC separated products with equal sensitivity,
- set up using available, or readily prepared, substrates and enzymes which are able to survive storage.

The carboxypeptidase reaction may be monitored by quantitative determination of the liberated D-alanine [Frére *et al.*, 1976]. However, this method was not suitable for high-throughput screening since the chemical estimation of liberated D-alanine requires a series of preparative steps and the alternative enzymic

a) *E. coli* Transpeptidase Activity



b) *E. coli* Carboxypeptidase Activity

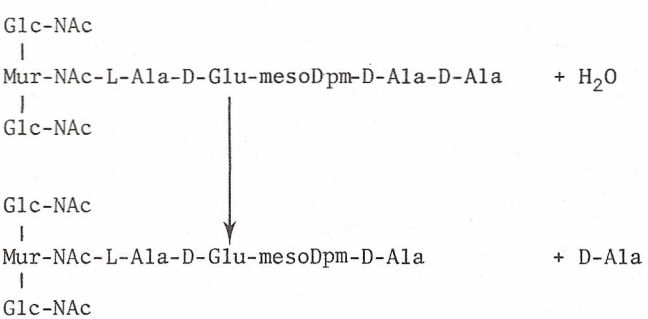


Fig. 2 Carboxypeptidase and Transpeptidase reactions in *E. coli* [Based on Izaki *et al.*, 1968].

determination involves a coupled reaction using D-amino acid oxidase and peroxidase. The possibility of using UDP-pentapeptide labelled in the terminal D-alanine residue was discounted because of the relative cost and difficulty of preparing sufficient quantities for high-throughput assays.

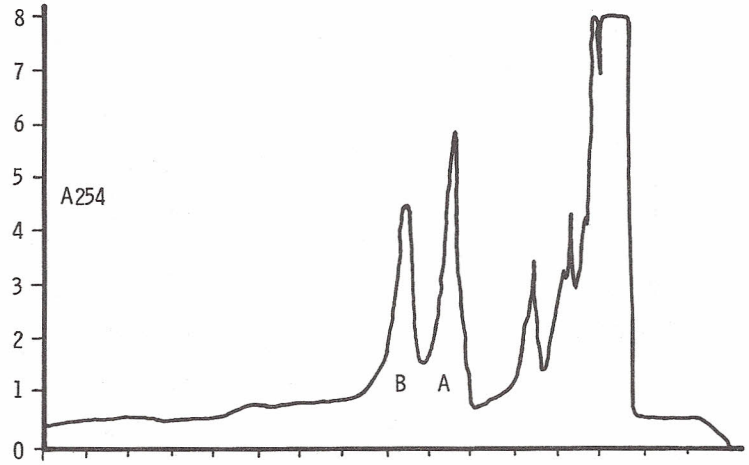


Fig. 3 HPLC separation of substrate (A-UDP-Pep5) and product (B-UDP-Pep4) of the carboxypeptidase reaction.

A novel assay for this enzyme was therefore developed using HPLC separation of the UDP-tetra- and tri-peptides from the unreacted substrate. Substrate and products were monitored by the absorbance of uridine nucleotide at 254 nm (Fig. 3). This method allowed an accurate estimation of tri- and tetra-peptide products as well as the unreacted substrate after separation from the interfering components of culture broths. Furthermore, by using automated sample injection and analysis the large numbers of samples required for a high-throughput screen could be handled.

The source of enzyme will affect the selectivity and sensitivity of the screens to known β-lactam antibiotics. The I_{50} values for a number of β-lactams to the enzymes prepared from *Actinomadura* R39, *E. coli* and *B. subtilis* (Table 5) showed the *Bacillus* enzyme to be the least sensitive (except towards penicillin N). A second important parameter is the effect of broth components on the test system. In contrast to *Actinomadura*, the *E. coli* enzyme was inhibited by a number of unfermented media (Table 6). The absence of non-specific inhibition, and also its resistance to proteolysis, combined to make the *Actinomadura* enzyme a good choice for the β-lactam primary screen. The sensitivity of the test was further improved by both purification of the enzyme (Table 7) and preincubation of the enzyme with the inhibitor. The

Actinomadura R39 carboxypeptidase is a stable enzyme that can detect pg quantities of β-lactam antibiotics. This enzyme was therefore tested for its ability to detect β-lactam producing *Streptomyces* spp. isolated from soils.

Detection of β-lactams in streptomyces spp. The use of standard β-lactam producing streptomycetes confirmed the potential of the carboxypeptidase assay for the detection of β-lactams in culture broth supernatants. A variety of cultural conditions was used to promote the expression of individual and complex groups of most of the known β-lactams (Fig. 4) by eight *Streptomyces* spp. (Table 8).

When used to screen the bioactive metabolites of 10,047 actinomycete isolates, producers of the described β-lactams were detected (Table 9). Of these, olivanic acid producing organisms constituted the vast majority (382). Penicillin N and cephamycins/cephalosporins were produced by 3 and 5 isolates respectively. Four new isolates (0.04% of the total screened) were found to produce new β-lactams and these are currently under investigation.

Conclusions

Increasingly the target directed screening approach is being adopted, not only in the search for new anti-microbial agents but for antifungal, antitumour, antiviral, anti-protozoal and pharmacologically active compounds. In this review the importance of new methods for the isolation, preservation and cultivation of antibiotic producing strains of microorganisms has not been discussed, but novelty in these areas is as important as in screening methods.

In concluding, one might ask the question "do we need new antibiotics"? In spite of the success achieved by antibiotics of microbial origin, and by the semisynthetic antibiotics, in solving the most urgent problems of chemotherapy, there are still a number of areas where new types of antibiotics with different spectra of activity and new mechanisms of action are required, for example to combat multiply resistant strains, systemic mycoses, allergic reactions and other toxic side effects. We will no doubt see significant advances in chemotherapy based on natural products from microorganisms.

Acknowledgements

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TABLE 5

Inhibition of carboxypeptidase by β-lactam antibiotics

Partly purified carboxypeptidase preparations obtained from cultures of *Actinomadura* R39 (Batch DEAE cellulose purified) [Frére *et al.*, 1974], *E. coli* (membrane bound) [Pollock *et al.*, 1972] and *B. subtilis* (membrane bound) [Blumberg and Strominger, 1972] were inoculated in a final volume of 30 µl with UDP pentapeptide (10 µmoles) and 10-fold serial dilutions of β-lactam antibiotics. After 1 h at 37°C the reaction was stopped by the addition of acetic acid (3 µl) and the DD-carboxypeptidase products determined by HPLC. Enzyme concentrations were selected to yield 4-6 nmoles of product in uninhibited controls. The concentration of β-lactam required to inhibit the enzyme activity by 50% (I_{50}) was determined from plots of $\log_{10}$ β-lactam against enzyme activity.

β-lactam antibiotic	Carboxypeptidase I_{50} (µg ml^{-1})		
	Actinomadura R39	*E. coli*	*B. subtilis*
Desacetoxy-cephalosporin C	1.7	32.3	102
Cephalosporin C carbamate	0.2	0.32	15
Cephamycin C	0.005	0.007	0.04
*Penicillin N	0.9	0.66	0.28
Clavulanic acid	4.5	2.8	18.9
†Thienamycin	1	131	105
Decarboxy-clavulanic acid	$>>10^3$	-	-
Nocardicin A	$>10^3$	-	-

* Approximately 50% pure

† Crude extract from *S. cattleya* NRRL 8057, results expressed relative to R39 enzyme I_{50}.

TABLE 6

Effect of media on E. coli *and* Actinomadura *R39 carboxypeptidase activity*

Carboxypeptidase activity was determined after incubation for 1 h with fresh unfermented media (18 µl) and UDP pentapeptide substrate as described in Table 5. The % inhibition of enzyme activity was determined relative to the water control.

Media	Carboxypeptidase Preparation			
	E. coli		*Actinomadura*	
	product (nmoles)	% inhib.	product (nmoles)	% inhib.
Water	5.7	0	4.5	0
Thienamycin E	3.5	38	-	-
Lilly 6	2.5	57	4.2	7
Lilly 1	4.5	21	4.2	7
Lilly 4	3.2	45	4.3	4
Jones 5	5.8	0	-	-
Beechams F	1.7	71	4.3	4
Yeast Peptone	4.4	22	4.5	0
Holomycin	-	-	4.4	2

TABLE 7

Sensitivities to β-lactam antibiotics of crude and purified Actinomadura *R39 carboxypeptidase*

The I_{50} determined as in Table 5 but using the β-lactamase-free *Actinomadura* R39 carboxypeptidase purified by elution from DEAE cellulose [Frére *et al.*, 1974] is given as a percentage of the I_{50} values obtained with partly-purified enzyme, from Table 5.

β-lactam	Relative Sensitivity (%)
Desacetoxy cephalosporin C	113
Cephalosporin C carbamate	113
Cephamycin C	103
Penicillin N	132

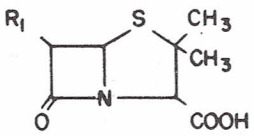
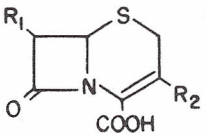
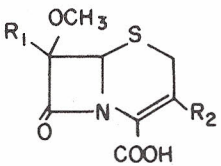
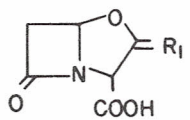
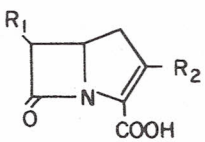
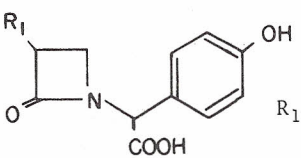
β-lactam type	Structure	Example
Penicillin		Penicillin N R_1 = α-amino-adipic acid (D)
Cephalosporin		Cephalosporin C Carbamate R_1 = α-amino-adipic acid (D) R_2 = CH_2-O-CO-NH_2
Cephamycin		Cephamycin C R_1 = α-amino-adipic acid (D) R_2 = CH_2-O-CO-NH_2
Clavams		Clavulanic acid R_1 = CH.CH_2OH
Carbapenems		Thienamycin (trans β-lactam) R_1 = CH(OH)-CH_3 (OH) R_2 = S-CH_2-CH_2-NH_2 MM22380 (cis β-lactam) R_1 = CH(OH)-CH_3 R_2 = S-CH_2-CH_2-NHCOCH_3
Nocardicin		Nocardicin A R_1 = C(=O)-C(=NOH)-  -CH_2-CH_2-CH(COOH)-NH_2

Fig. 4 The β-lactam nuclei produced by Actinomycetes.

TABLE 8

Cultures of known producers of β-lactams with activity against R39 carboxypeptidase

Streptomyces sp.	β-lactams produced in culture broths				
	Cephamycins	Cephalosporins	Thienamycins Olivanic acids	Clavulanic acids	Penicillin N
S. griseus	+				
S. heteromorphus	+				
S. lipmanii	+		+		+
S. clavuligerus	+	+		+	+
S. jumonjinensis	+			+	
S. lactamdurans	+				
S. olivaceus			+		
S. flavogriseus			+		+
S. cattleya	+		+		

TABLE 9

*Results of a preliminary screen for β-lactams**

Number of isolates tested	10,047
Number of positives	394
Olivanic acids/Thienamycins	382
Penicillin N	3
Cephamycins/cephalosporins	5
Novel β-lactams	2 (+2)†

* All of the cultures detected produced a complex mixture of β-lactams and are categorized according to the major β-lactam.

† Two cultures produced minor quantities of novel β-lactams and are categorized under cephamycin/cephalosporin types. In the remaining 2 cultures the new β-lactams were predominant.

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