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Combinatorial chemistry in anti-infectives research

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The ever-increasing resistance to current anti-infective drugs has become a major concern to the medical community. As a result, research efforts have been stepped up with the ultimate goal to provide new, more effective and safer antimicrobial treatments that will overcome the resistance problem. In this context, advances in molecular biology, automation and combinatorial chemistry will play a crucial role in the timely introduction of these products onto the market. How the application of combinatorial techniques can impact anti-infectives research will be reviewed using illustrative examples.

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▼ Over the past decade, drug resistance has become a growing problem in the treatment of infectious diseases caused by bacteria, fungi, parasites and viruses. In particular, resistance of bacterial pathogens to current antibiotics has emerged as a major health problem. This is especially true in hospitals and chronic care facilities, which provide strong selection pressure for the emergence of resistance because of the large quantities and the variety of antibiotics used in these environments. As a result, infections such as pneumonia, meningitis and tuberculosis that would once have been easily treated with antibiotics are no longer so readily treated. Hospital-acquired infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) are especially difficult to treat.

At present, all widely used antibiotics, including some of the newer agents, such as the streptogramins and the new-generation fluoroquinolones, are subject to bacterial resistance. The urgent need to tackle the resistance problem and the lack of a robust pipeline of innovative antimicrobial substances has led to a dramatic increase in antibacterial research in academic, government and industrial laboratories. There are currently four principal approaches being pursued to challenge antibiotic resistance and identify new antibacterial drugs:

- Screening of compound libraries;
- modification of known antibiotics;
- protection of known classes by resistance mechanism inhibitors; and
- discovery of new agents through rational selection of novel targets underpinned by genomics.

Recent developments in combinatorial chemistry, molecular biology and robotics have had an enormous impact on the drug discovery process. Importantly, the role of combinatorial chemistry as a tool for accelerating the lead discovery as well as the lead optimization phase has now been firmly established. Numerous examples of the successful application of both solid- and solution-phase combinatorial techniques in the generation of active compounds against biologically relevant targets have been documented [1].

Elegant combinatorial strategies have been devised for the identification of potent antiviral [2–4] and antifungal [5,6] agents. However, the majority of reports in the anti-infectives area detailing combinatorial lead discovery and optimization approaches relate to the field of antibacterial research. In the following discussion, recent highlights will be presented that show the merits of solid-phase and solution-phase parallel synthesis in the search for new antibiotics.

Screening of compound libraries

Historically, screening for novel antimicrobial compounds has been performed by testing large libraries of natural products for their ability to kill bacteria and has led to many of the antibiotics used today. This classical approach is undergoing renewed interest because of the availability of diverse libraries

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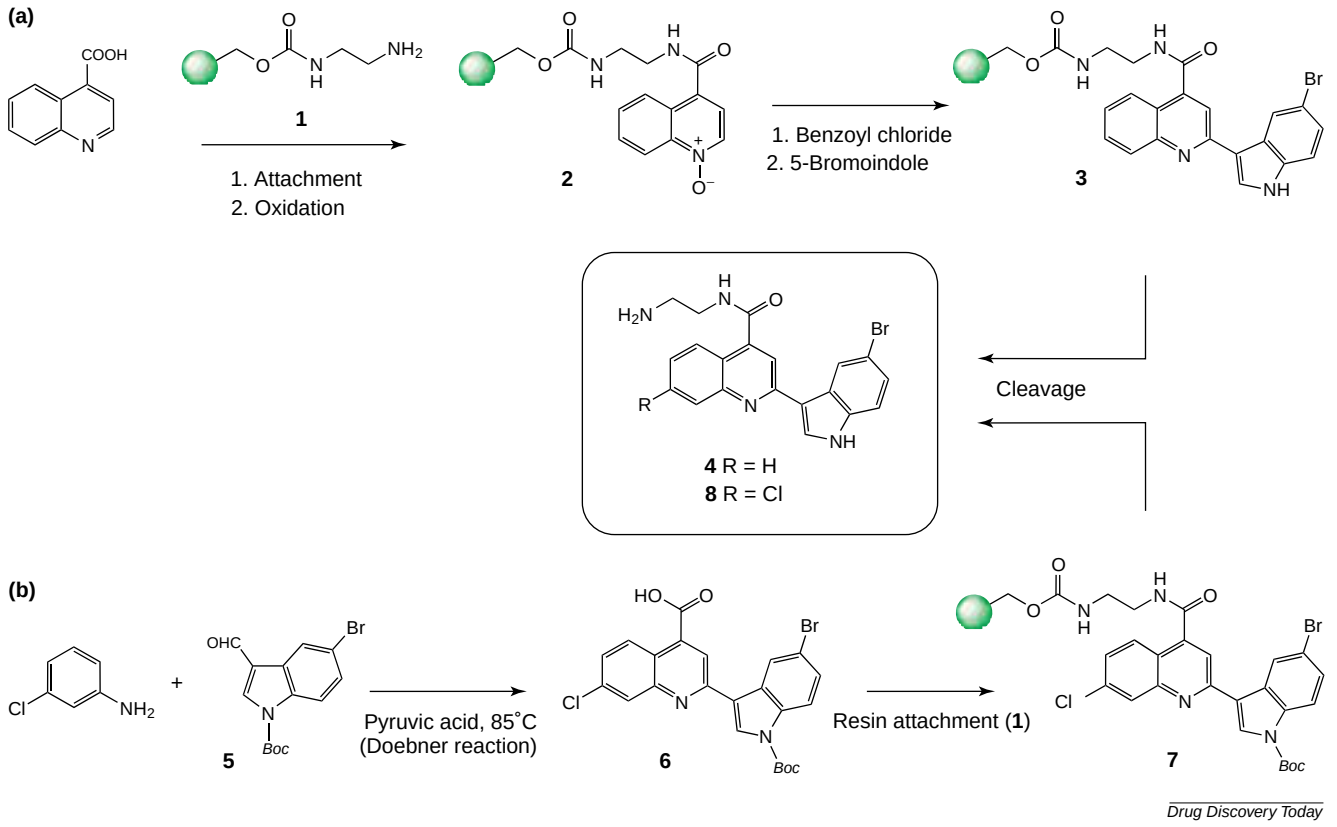


Figure 1. Synthesis of 2-(1*H*-indol-3-yl)quinoline derivatives (*Boc* = *tert*-butyloxycarbonyl protecting group). Compounds **4** and **8** (boxed) are the target compounds in this scheme.

prepared through combinatorial chemistry and advances in technology enabling high-throughput screening. However, this strategy has disadvantages, such as low sensitivity and the fact that the targets of the respective compounds are unknown.

Despite these drawbacks, screening of large mixture-based combinatorial peptide libraries led to the successful identification of new antimicrobial compounds, as reviewed by Blondelle and Houghten [7]. Since then, emphasis has shifted towards the preparation and testing of smaller, focussed libraries of low molecular weight compounds. Examples include the discovery of trisubstituted furanones [8], 1,2,4-triazolo[3,4-*b*]-1,3,4-thiadiazepines [9], sulfonamido hydroxamic acids [10], hydrazinyl ureas [11] and 1,5-dialkyl-2,4-dinitrobenzene derivatives [12] as potent antibacterial leads. Notably, researchers at Sepracor (Marlborough, MA, USA) identified a structurally novel class of antimicrobials that are active against MRSA, by screening a focussed library of 2-(1*H*-indol-3-yl)quinolines [13]. An initial library was prepared using solid-phase *N*-oxide chemistry, as illustrated by the synthesis of compound **4** (Fig. 1a). 1,2-Diaminoethane immobilized to 4-(hydroxymethyl)phenoxymethyl polystyrene (**1**) was functionalized with quinoline-4-carboxylic acid and subsequently oxidized to produce compound **2**. The resin was then reacted sequentially with benzoyl chloride and 5-bromoindole (producing compound **3**), and ensuing treatment with trifluoroacetic acid in dichloromethane yielded compound **4**.

Screening the library, which contained three compounds per well, against a representative panel of microorganisms and subsequent deconvolution, identified the 2-(1*H*-indol-3-yl)quinoline derivative **4** as the most potent compound against MRSA and VRE; with a minimal inhibitory concentration (MIC) of 12.5 μg ml^{-1} and 25 μg ml^{-1}, respectively.

To assess the structure–activity relationship (SAR) of the 2-(1*H*-indol-3-yl)quinoline template, a revised, more flexible combinatorial strategy was used based on the Doebner quinoline synthesis [14]. A representative example of this approach is the synthesis of derivative **8** (Fig. 1b), which was obtained in three successive steps: (1) reaction of chloroaniline with indole carboxaldehyde **5** in the presence of pyruvic acid (producing compound **6**); (2) coupling to polymer-bound diamine **1** (producing compound **7**); and (3) trifluoroacetic acid-mediated cleavage (producing compound **8**).

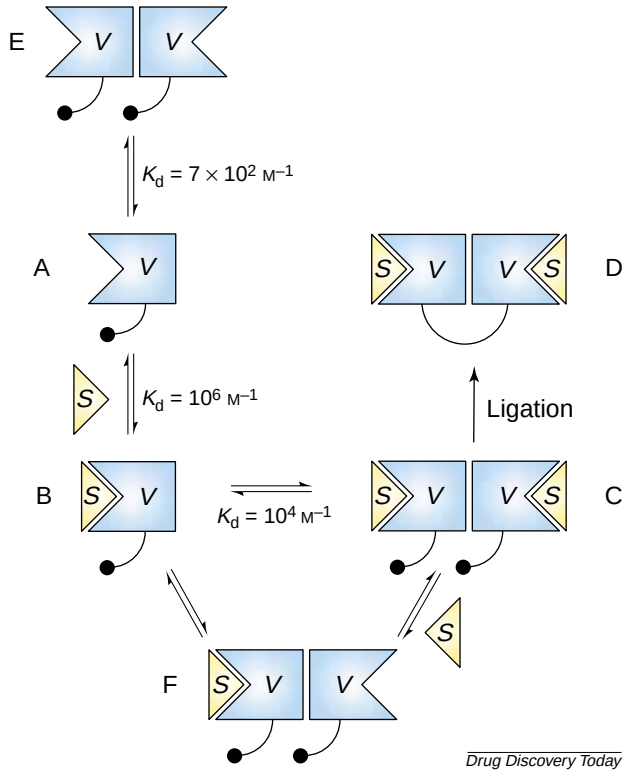


Figure 2. Schematic representation of target-accelerated combinatorial synthesis of vancomycin dimers. A library of vancomycin analogues (*V*, blue shape) is incubated with their target substrate, Ac_2-L-lys-D-Ala-D-Ala (*S*, yellow shape). Because the formation constant of the vancomycin dimer (K_d ~7×10^2 M^{-1}) is greater in the presence of its substrate (K_d ~10^4 M^{-1}), it is expected that the target-bound dimer assembly (C) should ligate faster than its non-target-bound counterpart (E).

Compound **8** showed a 16-fold improvement in MRSA and VRE activity compared with compound **4**, and good *in vitro* potency was obtained against two clinical isolates of glycopeptide intermediate-resistant *S. aureus* (MIC 0.39 and 0.78 μg ml^{-1}).

Interestingly, preliminary mode-of-action studies suggested that some quinoline indoles act as inhibitors of cytoplasmic membrane energization, leading to both rapid inhibition of macromolecular synthesis and cell lysis, whereas other compounds appeared to promote non-lytic killing of *S. aureus* by inhibition of RNA synthesis [15].

Modification of known antibiotics

The widely recognized importance of β-lactams as antimicrobial drugs has prompted the development of solid-phase and combinatorial approaches towards their synthesis [16]. Other well-known antibiotics that have proven their value in the clinic, including the fluoroquinolones [17], macrolides [18] and aminoglycosides [19], and have also been shown to be amenable to combinatorial synthesis. Furthermore, solid-phase strategies have been used in the identification of analogues of the newly approved antibiotic linezolid, showing potent *in vivo* antibacterial activity [20].

Considerable efforts in the field of anti-infectives research have been devoted to identify analogues of vancomycin. Vancomycin, a prominent member of the glycopeptide class of antibiotics, has been used clinically for the past 40 years to treat infections caused by Gram-positive bacteria. Its renowned action against MRSA bacteria has made it the antibiotic of last resort. However, the emergence of VRE and vancomycin-intermediate susceptible *S. aureus* (VISA) has prompted renewed and vigorous research targeting modified vancomycin analogues with restored activities against VRE or VISA.

The antibacterial activity of vancomycin arises from its ability to inhibit peptidoglycan biosynthesis within the bacterial cell wall. Specifically, vancomycin binds with high affinity and specificity to the C-terminal L-lysyl-D-alanyl-D-alanine fragment of the growing peptidoglycan biosynthetic precursor through an intricate network of hydrogen bonds, thereby inhibiting cell wall growth and crosslinking.

A second molecular recognition function of glycopeptides is that of self-association into homodimers. This phenomenon has been correlated with enhancements in *in vitro* antibacterial activity, which is consistent with a model in which dimerization increases the affinity of glycopeptides for their peptidoglycan precursor targets [21]. Importantly, covalent dimerization of vancomycin via the C-terminus was shown to give antibacterial potency against VRE [22].

An interesting combinatorial approach exploring the potential of covalent dimers to overcome vancomycin resistance, was reported by Nicolaou and colleagues [23]. Key to their strategy was the self-assembly of monomers in the presence of the target and subsequent dimerization of the most stable and longest-lived supramolecular assemblies using a latent reactive functionality. A schematic representation of this so-called 'target-accelerated combinatorial synthesis' (TACS) is outlined in Fig. 2.

Monomer building blocks were obtained using both solution- and solid-phase parallel chemistry. Covalent dimerization of the appropriately functionalized vancomycin monomers was achieved across the saccharide domain using the olefin metathesis reaction or disulfide bond formation. These ligation methods, which can operate efficiently at biologically relevant temperatures and in aqueous media, are compatible with the polyfunctional structure of vancomycin. A key attribute is that they operate reversibly, thereby enabling equilibration.

Following initial studies in which the optimal tether and substrate was established, an eight-component TACS experiment was performed applying the olefin metathesis reaction. Out of the expected 36 library members, only 30 were observed by mass spectrometry as a consequence of the degeneracy of six of the dimers. On the basis of the observed relative abundance of the vancomycin dimers, 11 compounds were individually resynthesized. Subsequent screening for antibacterial activity showed that the TACS experiment predicted the overall trend of the observed biological activity of the library members relatively reliably. More importantly, several highly potent antibiotics were identified showing activity against both vancomycin-susceptible and vancomycin-resistant bacteria.

Resistance mechanism inhibitors

Drug inactivation, target modification and alteration of target accessibility through drug efflux and decreased uptake, are the three primary mechanisms that microorganisms use against antimicrobial therapy. β-Lactamase inactivation of β-lactams is the most prominent example of resistance because it involves inactivation of the drug. Carbapenems, such as imipenem and meropenem, are β-lactam antibiotics noted for their broad spectrum activity and stability to most β-lactamases. However, bacterial resistance to these clinically important antibiotics is on the rise, partly because of the increasing incidence of class B metallo-β-lactamases (MBLs) [24]. Unlike the more common serine β-lactamases, MBLs are active-site zinc enzymes, which efficiently hydrolyze carbapenems as well as penicillin and cephalosporin members of the β-lactam family, and occur primarily in Gram-negative bacteria. One approach that could overcome this important medical problem is to combine the antibiotic with an enzyme inhibitor, as was successful with Augmentin™ (GlaxoSmithKline; Research Triangle Park, NC, USA), which comprises the β-lactam antibiotic amoxicillin, and clavulanic acid – a suicide inhibitor of the class A serine-β-lactamases. Unfortunately, serine β-lactamase inhibitors such as clavulanic acid or sulbactam do not inactivate MBLs.

Of the MBLs identified to date, the IMP-1 enzyme appears to pose the greatest threat to antimicrobial chemotherapy because of the transferable nature of its gene. Previously, thiodepsipeptides have been reported to be inhibitors of MBLs (including IMP-1), but with widely varying potencies [25]. To identify IMP-1 inhibitors that have the potential to overcome IMP-1-mediated resistance in bacteria when administered with a β-lactam antibiotic, researchers at Merck (Rahway, NJ, USA) have devised a solid-phase synthesis of thioester and thiol derivatives **13** and **14**, respectively (Fig. 3) [26].

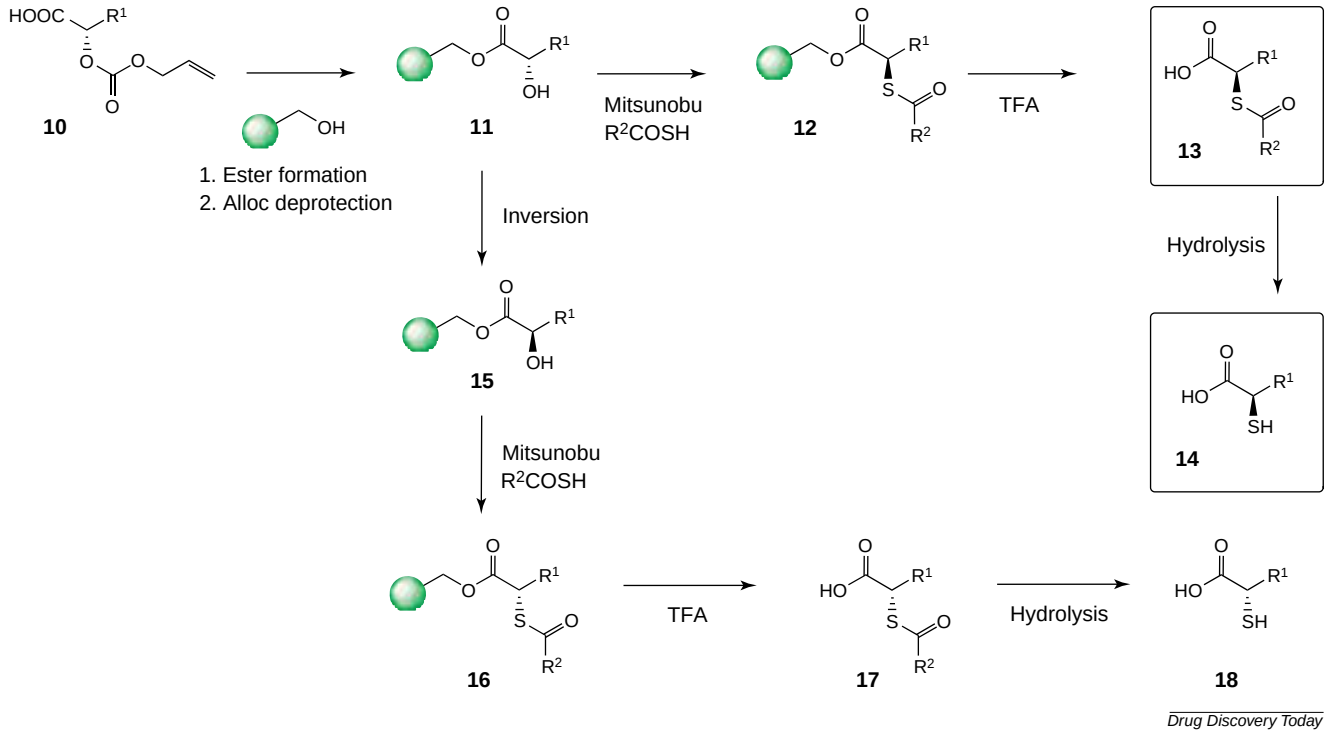
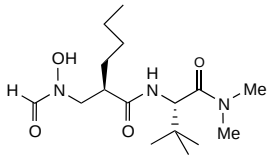
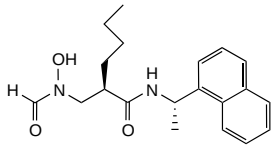
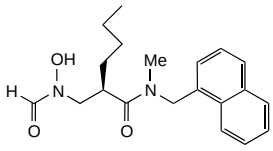
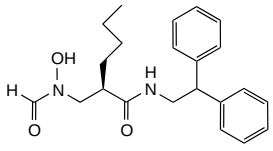
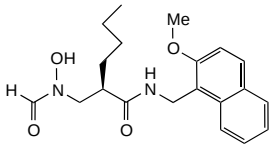


Figure 3. Combinatorial synthesis of metallo β-lactamase inhibitors. Compounds **13** and **14** (in boxes) are the target compounds in this scheme.

Table 1. Enzyme and *in vitro* antibacterial activity for selected PDF inhibitors

Structure	Compound[a]	*Escherichia coli* PDF IC_{50} (nM)	*Staphylococcus aureus* MIC (μg ml^{-1}) (5)[b]	*Streptococcus pneumoniae* MIC (μg ml^{-1}) (3)	*Haemophilus influenzae* MIC (μg ml^{-1}) (2)
	BB3497	7	4–32	8–8	<0.015–0.25
	**21a**	30	8–2	8–8	1–2
	**21b**	20	0.25–2	4–8	2–16
	**21c**	60	0.125–0.5	4–8	2–8
	**21d**	20	1–2	4–8	0.5–1

[a]For synthesis of **21a–d** see Figure 4.
[b]Strains (number of strains in parenthesis): *S. aureus* ATCC 29213 MSSA, ATCC 25923 MSSA, ATCC 6538 MSSA, TN1152 MRSA and TN 3601 MRSA; *S. pneumoniae* 2403 PSP, 2391 PIP and 2390 PRP; *H. influenzae* 1390 and 1414.

The target compounds were synthesized using a modified Mitsunobu reaction of a resin-bound α-hydroxy ester (**11**) and thioacetic acid or thiobenzoic acid as the key step. The starting protected α-hydroxy acids **10** were prepared in solution via an asymmetric hydroxylation protocol. After attachment of substrate **10** to the resin, removal of the allyloxycarbonyl protecting group provided derivative **11**. A subsequent Mitsunobu reaction with thioacetic acid or thiobenzoic acid gave solid-supported thioesters **12**. After cleavage from the resin, thioesters **13** were obtained in 80–90% yield and >95% purity by high performance liquid chromatography (HPLC). The acyl group was then removed with aqueous ammonia under minimal racemization conditions to produce thiols **14**. Compounds epimeric at the thiol centre were prepared from the epimeric resin-bound α-hydroxy ester **15**, which was obtained either by starting with the enantiomer of α-acyloxy acid **10** or by inverting the hydroxy centre of **11**.

Evaluation of the target compounds for activity against IMP-1, indicated that both thioesters **13** and thiols **14** were potent inhibitors of the enzyme with IC_{50} values in the nanomolar range, whereas the corresponding enantiomers **17** and **18**, respectively, were considerably less active [26]. Furthermore, it was shown that introduction of more hydrophobic R^1 substituents increased enzyme activity.

Importantly, additional studies demonstrated that thioesters **13** increased the sensitivity of an IMP-1-producing laboratory strain of *Escherichia coli* to the carbapenem L742728 [27]. However, it was noted that the combination was less effective against IMP-1-producing *Serratia* and *Pseudomonas* strains, highlighting the need to overcome permeability barriers in Gram-negative bacteria.

Novel antibacterial targets

Genomics is regarded by many in the pharmaceutical industry as the route to novel antibacterial agents. However, to date, there have been few published successes in terms of identifying antimicrobial agents. Novel bacterial targets that have been examined on the basis of their requirement for or contributions to susceptibility *in vitro*, include lipid A

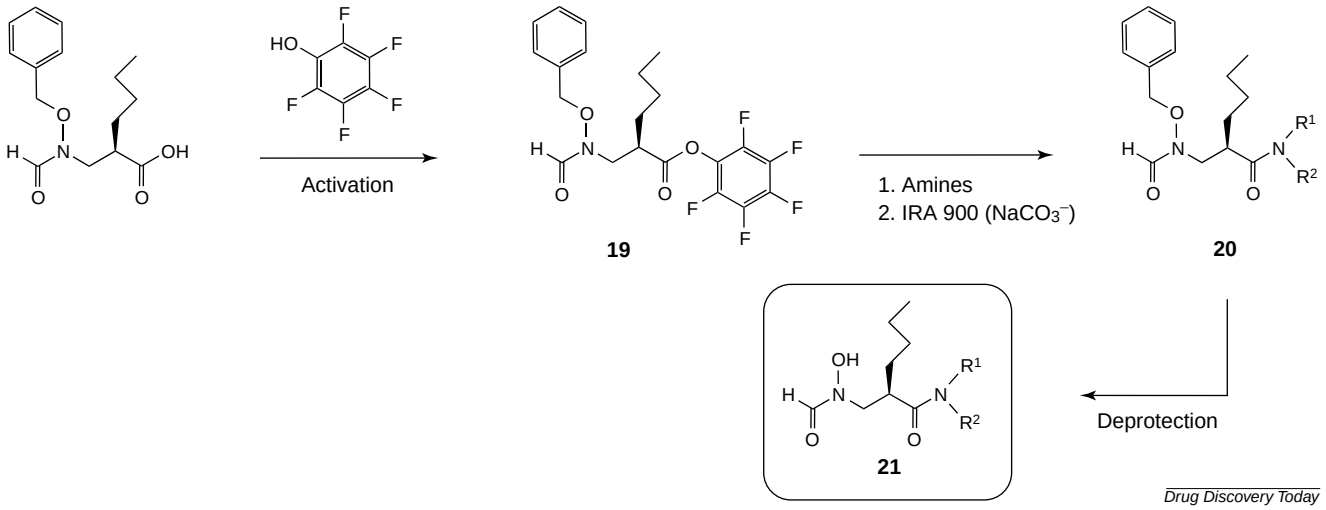


Figure 4. Solution-phase parallel synthesis of polypeptide deformylase (PDF) inhibitors. Compound **21** (boxed) is the target compound in this structure.

biosynthesis [28], two-component regulatory systems [29] and, in particular, polypeptide deformylase (PDF) (Ref. [30]).

PDF is a bacterial metalloenzyme that specifically deformylates newly synthesized peptides as an essential part of their post-translational processing. The gene encoding PDF (*def*) is present in all sequenced pathogenic bacterial genomes, and protein synthesis in the eukaryotic cytoplasm does not involve the formylation–deformylation mechanism. PDF inhibition therefore has the potential to generate a new class of broad-spectrum antibacterial agents devoid of established resistance mechanisms.

Recently, British Biotech described the *in vitro* and *in vivo* antibacterial properties of the *N*-formyl-hydroxylamine PDF inhibitor BB3497 (Table 1), demonstrating the potential of this new class of antibiotics [31]. For the identification of compounds with improved antibacterial activity and pharmacokinetic (PK) properties , non-peptidic compounds were prepared in which the *tert*-leucine dimethyl amide fragment was replaced with a large variety of amine substituents [32]. Virtual screening was used as a tool to optimize target compounds for PDF enzyme binding. Molecules that did not demonstrate favourable interactions with the PDF crystal structure were not pursued. In addition, amine fragments were screened *in silico* to enable maximum diversity.

Parallel synthesis of non-peptidic BB3497 analogues was achieved via a solution-phase protocol (Fig. 4), which involved: (1) formation of the left-hand-side pentafluorophenyl ester (to produce compound **19**); (2) reaction with a diverse selection of amines (to produce compound **20**); and (3) removal of the benzyl protecting group under transfer hydrogenation conditions (to produce compound **21**). A simple purification protocol was followed for effective scavenging of displaced pentafluorophenol using Amberlite IRA 900 ($NaCO_3^-$-form; Fluka, Buchs, Switzerland) ion-exchange resin. The average purity of the compounds was 85–95% by HPLC.

All compounds were screened against the PDF enzyme as well as a relevant Gram-negative and a Gram-positive test organism. The results showed that the PDF enzyme tolerated a diverse range of right-hand-side functionalities with IC_{50} values as low as 7 nM [32]. By contrast, only a relatively small set of compounds displayed MICs in the low micromolar range. The lipophilicity of these compounds appeared to be essential for antibacterial activity, which could not be explained by enzyme inhibition alone. Additional testing against clinically relevant bacterial pathogens revealed derivatives **21b** and **21c** (Table 1) as potent antibacterials with good activity against both drug-sensitive and drug-resistant strains of *S. aureus*. Unfortunately, both compounds showed less favourable PK properties than the original lead compound BB3497.

It is important to note that the target-directed approach, as highlighted by the previous example, does not necessarily provide good antibacterial agents. Indeed, other optimized PDF enzyme inhibitors have been described that lack the expected *in vitro* antibacterial activity [33]. These observations can be rationalized in terms of the compounds' inability to penetrate the bacterial outer membrane and/or the existence of active efflux systems.

Concluding remarks

Combinatorial chemistry has become an invaluable addition to the toolkit of the medicinal chemist. It encompasses

an ever-growing range of chemistries and techniques that have the potential, if applied sensibly, to deliver more drug-like compounds in a timely fashion. As exemplified in this review, well-designed combinatorial strategies can play an important role in the discovery and rapid optimization of potent new antibiotic leads, and potentiators of antimicrobial action required to overcome bacterial resistance. Significantly, the examples outlined here used techniques that are applicable to other areas within the field of medicinal chemistry. In particular, target-accelerated combinatorial synthesis provides a challenging concept that might well prove its value in the identification of inhibitors of other relevant biological targets.

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