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Analysis of the Effectiveness of Different Biosynthetic Techniques in Altering Natural Products to Create Novel Antibiotics

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Abstract: Antibiotics are ubiquitous in modern medicine due to their usefulness in combating sickness and disease. They vary greatly in structure and function, permitting their use in the treatment of a wide variety of bacterial infections. However, due to the development of resistance in bacteria, antibiotics must constantly be updated to remain effective. There are many strategies that have been applied to alter natural products and subsequently create novel antibiotics, including precursor-directed biosynthesis, genetic engineering, and combinatorial biosynthesis. Different techniques have been proven effective on different natural products due to the diverse structures of these natural products, each ranging in effectiveness. This review compiles several studies where naturally occurring β-lactams and glycopeptides have been modified through the previously mentioned techniques to create new antibiotics. The studies impart insight into the effectiveness of each of these techniques and their potential utilization in the future of the medical field.

Author keywords: Antibiotics; natural products; precursor-directed biosynthesis; genetic engineering; combinatorial biosynthesis

Introduction

Medicine, an essential aspect of everyday life, has played its part in human society for thousands of years. A common resource for the creation of medicine is nature, an abundant utility that can be found around humans at all times. Natural sources such as plants, animals, fungi, and bacteria are all complex organisms that produce diverse metabolites with intricate chemical structures, and they all can and *have* been used to create new medicine in a variety of ways.

Natural products can be described as small organic molecules produced via conditional pathways that control the structure and, therefore, function of the molecules. Around 500,000 natural products are recorded in the literature, 70% of which originate from plants.[1] Natural products are a keystone of drug discovery and are commonly employed in the production of pharmaceuticals, whether to use directly or to derive new medicine from a natural product base. In cancer research between the 1940s and 2014 alone, 49% of small molecules were natural products or were derived from them.[2] The structure of a natural product is essential to its pharmaceutical function, as different structures allow for different interactions and binding affinity between enzymatic substrates and their targets. Due to the diversity in structure found across these thousands of natural products, they are ideal for the creation of a multiplicity of different medicines all used for various purposes.

More specifically in the medical realm, natural products have been utilized to construct antibiotics. Antibiotics are a type of medicine used to fight bacterial infection, whether by killing specific bacteria infiltrating the body (bactericidal) or stunting the bacteria's ability to reproduce (bacteriostatic).[3] The first antibiotic was discovered in 1928 by Alexander Fleming, who observed that a mold of the *Penicillium* fungus was preventing the growth of the bacteria *Staphylococcus* in a petri dish. Fleming's discovery was life changing—the natural product produced by the *Penicillium*, which was determined to be the source of the bactericidal activity, was then used to create penicillin, the first antibiotic.[4]

Fleming's method of discovering *Penicillium*'s antibiotic properties is also used today to identify antibiotic activity and test the effectiveness of antibiotic medication. Although Fleming's breakthrough was accidental, W. M. M Kirby and A. W. Bauer eventually developed the Kirby–Bauer Disk Diffusion Susceptibility Test Protocol, which is based off on the Fleming's method of discovering *Penicillium*'s antibiotic activity. In this protocol, bacterial isolates are spread evenly on an agar plate to create a lawn of growth, and antibiotic-impregnated paper disks are placed on the surface. After incubation, the plates are examined for clear zones of inhibition around the disks, indicating susceptibility. In contrast, the diameters of these zones are measured to determine the bacterial resistance or sensitivity to the tested antibiotics. While Fleming found that the mold itself

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was causing these zones of inhibition, indicating the presence of antibiotics through their activity, the Kirby-Bauer method highlighted further information about the antibiotic activity.[5] The effectiveness of antibiotics can also be tested using other processes, such as the broth dilution test. The broth dilution test is a method used to determine the minimum inhibitory concentration (MIC) of an antibiotic, which is the lowest concentration that inhibits the visible growth of a microorganism. In this test, a series of decreasing antibiotic concentrations are prepared in the broth culture. The microorganism is then added to each concentration, and after incubation, the MIC is identified as the lowest concentration where no microbial growth is observed.[6]

Because antibiotics, like other medicines, are commonly created using natural products, the influence structure has on function is apparent in antibiotics, as well. For example, β-lactams, a group of antibiotics uniquely comprising a β-lactam ring—a ring of three carbons and a nitrogen atom in which one of the carbons bonded to the nitrogen atom is additionally double bonded to an oxygen atom—target bacteria by inhibiting their cell wall biosynthesis.[7] Penicillin is an example of a β-lactam. Another example of an antibiotic natural product are the glycopeptides; however, their composition is very different from that of a β-lactam. Because β-lactams and glycopeptides, both antibiotics derived from natural products, have different structures, they have different functions, as well.

Scientists have observed the capabilities of natural products and have aimed to synthesize new medicine whilst using natural resources through biosynthesis. Biosynthesis is an enzyme-catalyzed process in cells of living organisms by which substrates are converted to more complex products.[8] There are a variety of ways in which antibiotics and other medicines can be biosynthesized. Some specific processes are substrate-directed biosynthesis, genetic engineering, and combinatorial engineering. These techniques, as well as a variety of other techniques, are crucial to learn for medical biochemists aiming to create new antibiotics.

Precursor-directed biosynthesis, or substrate-directed biosynthesis, combines chemical synthesis and enzymatic transformations, which allows non-native starting materials to be incorporated into biosynthetic pathways. Specific chemical precursors, or substances (such as substrates) that precede and serve as a starting material for the synthesis or production of another compound, are introduced to the microbial culture, creating targets that are structurally similar to the desired targets. Within the microorganism, enzymes recognize and process these precursors, converting them into complex molecules through sequential biochemical reactions. This method not only expands the range of compounds that can be produced but also enhances the efficiency and specificity of biosynthetic processes.[9]

Genetic engineering is a technique used to manipulate the genetic material of organisms, allowing scientists to alter DNA sequences and introduce new traits into an organism's genome. This process typically involves isolating specific genes responsible for desired traits, modifying them in the laboratory, and inserting them back into the organism's genome using tools such as restriction enzymes, DNA ligases, and vectors like plasmids. It enables precise control over genetic traits, accelerating advancements in biotechnology.[10]

Combinatorial biosynthesis is the process of creating new natural products by combining genetic elements from different biosynthetic pathways. By manipulating and combining genetic elements within microorganisms, scientists can generate libraries of structurally diverse molecules that would be challenging to produce through traditional chemical synthesis. Combinatorial biosynthesis involves engineering the genetic code of organisms to produce variations of natural products or entirely new compounds by reprogramming their metabolic pathways.[11]

It is critical to interpret the changes that can be made by these biosynthesis techniques and, ultimately, the effectiveness of different biosynthetic strategies (combinatorial biosynthesis, genetic engineering, precursor-directed biosynthesis, etc.) in altering natural products to create new antibiotics or improving the production of these natural antibiotics in fermentative culture.

Overview

β-*lactams*

β-lactams are a type of natural product thought to be first discovered when Fleming observed the production of penicillin, a type of β-lactam, through the mold *Penicillium*. Like penicillin, β-lactams typically come from natural sources. Several types of β-lactams exist, such as penicillins, cephalosporins, carbapenems, and so on. The base structure of a β-lactam consists of a ring made from three carbon atoms and a nitrogen atom; one of the two carbon atoms that are bonded with the nitrogen atom is also double bonded to an oxygen atom. The main target of a β-lactam is the bacterial cell wall; it impedes its biosynthesis by inhibiting the enzyme penicillin-binding protein, or transpeptidase, which is responsible for crosslinking of the peptidoglycan in the cell wall.[7]

The paper "Engineering the synthetic potential of β-lactam synthetase and the importance of catalytic loop dynamics" details the processes of using a combination of substrate-directed biosynthesis and genetic engineering to generate a new β-lactam product. In this paper, mutagenesis, a type of genetic engineering, was used. Mutagenesis is the process where an organism's deoxyribonucleic acid (DNA) changes, resulting in a gene mutation, which is a permanent and heritable change in genetic material. This change results in altered protein structure, function, and phenotypic changes.[12] The enzyme was engineered with the native β-lactam synthetase (βLS) enzyme through mutations to accept an altered substrate bearing an alkyl substituent. The study involved analyzing five X-ray structures of βLS bound with substrates, products, and non-reactive cofactor or substrate analogs, providing a detailed view of the catalytic cycle in which substrates bind, react, and yield products as surrounding residues move within the active site. Critical to this process were substrate pre-organization,

adenylation, and the precise placement of a catalytic dyad along with a critical lysine, all of which facilitated β-lactam formation. In order for the β-lactam to accommodate larger substrates, the diastereomeric preference of the V446A and V446G mutants of βLS were evaluated. The substitution of V446A in βLS created a sufficient amount of additional volume in the active site to achieve higher selectivity for one diastereomer over the other. In the βLS crystal structures, it was observed that the CEA binding to a substrate is facilitated by a backbone hydrogen bond from Gly349 to the carboxylate of the arginine moiety and by interactions with the guanidino group from Glu382, Asp373, and a water molecule. When the catalytic loop is open, the carboxyethyl portion of the substrate is free to sample the space around the active site, unlike when the catalytic loop is closed. The paper concluded that kinetics experiments linking structural and dynamic information to guide protein engineering are crucial in the field of protein engineering. Through mutagenesis, the authors were able to generate a new, methylated β-lactam which could have improved activity.[13]

Mutagenesis was also utilized in the study "Saturation mutagenesis reveals the importance of residues αR145 and αF146 of penicillin acylase in the synthesis of β-lactam antibiotics," along with substrate-directed biosynthesis.[14] More specifically, saturation mutagenesis was employed, in which codons are substituted with all possible amino acids at the position.[15] In the paper, saturation mutagenesis was used specifically on the enzyme penicillin acylase with the amino acids αR145 and αF146 to see what the effects would be. Substrate-directed biosynthesis was used as well as genetic engineering as penicillin acylase was given a native substrate, 6-APA, and a non-native substrate, phenylglycine amide, to create ampicillin. It was observed that most of the αR145 mutants in penicillin acylase exhibited a higher concentration of active enzyme in the cultivated cells than those expressing the wild-type enzyme. This indicated that the auto-catalytic maturation of the mutants is facilitated in the α145 mutants due to the altered active site, an observation that was not found for the mutants in position αF146. The best-performing R145 mutants, R145G, R145S, and R145L mutants, also demonstrated a more effective synthesis of β-lactam antibiotics than the wild-type penicillin acylase, resulting in an increased conversion of the natural product 6-APA to the non-natural antibiotic ampicillin by up to 16%, as well as to a decreased hydrolysis of the exogenous acyl donor by 29–56%, while the reduction in synthetic activity prominent in αF146 mutants was absent in the αR145 mutants. Mutant R145G revealed the best parameters for the synthesis of ampicillin, being able to produce 77 mM of ampicillin with a 29% reduction in the loss of the acyl donor due to hydrolysis as compared to the wild-type enzyme. Therefore, a single mutation in the targeted region has the potential to improve the catalytic properties of the enzyme for antibiotic synthesis. Moreover, when larger substrates like penicillin G were bound, both αR145 and αF146 residues were found to shift away from the acyl binding site in an induced fit mechanism. These findings indicate that αR145 and αF146 are particularly promising targets for improving the catalytic efficiency of penicillin acylases.[14] Further engineering and application of this enzyme could be used to biosynthesize other non-natural β-lactams from the natural core, 6-APA.

In comparing the effectiveness of each method as shown by the papers, both substrate-directed biosynthesis and genetic engineering allow for precise modifications to existing biosynthetic pathways; these modifications often improve pre-existing antibiotics. Substrate-directed biosynthesis does not implicitly require the enzyme itself to be altered, and therefore is generally less complex than genetic engineering; however, it has its limitations, as it is necessary for the enzyme to recognize and fit in with the altered substrate. Both approaches have shown significant promise in addressing antibiotic resistance, with genetic engineering providing greater diversity in its alterations and substrate-directed synthesis providing a less complex approach that may be quicker in implementation.

Glycopeptides

Glycopeptides, a different natural product, are actinomycete-derived antibiotics with unique tricyclic or tetracyclic heptapeptide cores that are usually glycosylated and sometimes have additional lipophilic fatty acid side chains.[16] Although, like β-lactams, glycopeptides also target cell way biosynthesis, specifically by inhibiting the synthesis of peptidoglycan by preventing the activity of the transpeptidase enzyme, β-lactams covalently modify the enzyme, rendering it inactive, while glycopeptides sterically block the enzyme from accessing its substrates.

There are several techniques for altering glycopeptide antibiotics, one of which includes enzymatic glycosylation. Glycosylation refers to a specific enzymatic process in which glycans are attached to lipids or proteins. Glycosylation is the most common and complex post-translational modification observed in the secretory pathway. Protein glycosylation is the result of enzymatic reactions which facilitate the addition of carbohydrates to proteins.[17] Enzymatic glycosylation is a method of attaching sugar moieties to various molecules using enzymes called glycosyltransferases.[18] Glycosyltransferases are typically seen as catalysts for unidirectional reactions. However, four glycosyltransferases that come from calicheamicin and vancomycin have the ability to catalyze reversible reactions, exchanging sugars and aglycons.[19] The study "Tandem action of glycosyltransferases in the maturation of vancomycin and teicoplanin aglycones: novel glycopeptides" found that two glycosyltransferases, GtfE and GtfD, work in tandem to add sugar moieties to the vancomycin aglycone. More specifically, GtfE and GtfD work sequentially to add and modify sugar moieties on the aglycone structures of vancomycin and teicoplanin. The first enzyme, GtfE, attaches a glucose molecule to the aglycone, and the second enzyme, GtfD, further modifies the sugar structure. These actions from the enzymes are crucial for the proper maturation of the antibiotic and its subsequent antimicrobial activity. High-performance liquid chromatography assays confirmed the activity of GtfE and GtfD, showing that GtfD catalyzes the formation of the disaccharide in vancomycin, while GtfA and GtfC play similar roles

in chloroeremomycin. Notably, GtfC, which is 69% identical to GtfD, forms the α-1,2-disaccharide on the heptapeptide scaffold, whereas GtfA, with 55% identity to GtfD, acts as the 4-*epi*-vancosaminyltransferase. Assaying GtfC or Gtfd needs the available NDP-β-L-4-epi-vancosamine or NDP-β-L-vancosamine as a cosubstrate, along with a nucleotide that is likely to be TDP or UDP. This enzymatic process results in the production of glycopeptide derivatives with altered sugar structures. The researchers were able to create novel glycopeptide derivatives by altering the substrates used in these reactions, demonstrating the potential for developing new antibiotics with improved properties.[20] Overall, this method of combinatorial and precursor-directed biosynthesis is much more productive than regular chemical synthesis, particularly for quickly studying the effect of sugar modifications on the central hydroxyphenylglycine-4 of glycopeptide antibiotics.[21]

In the paper "Mutasynthesis of Glycopeptide Antibiotics: Variations of Vancomycin's AB-Ring Amino Acid 3,5-Dihydroxyphenylglycine," the authors explored the use of mutasynthesis, a type of precursor-directed biosynthesis, to create novel glycopeptide antibiotics by modifying the AB-ring of vancomycin. Mutasynthesis is a technique where unnatural amino acids are incorporated into the antibiotic's structure to generate new derivatives. The authors utilized the Δ*dpgA* mutant of *Amycolatopsis balhimycina*, which is deficient in synthesizing 3,5-dihydroxyphenylglycine. By supplementing the medium with synthetic 3,5-dihydroxyphenylglycine analogs, they produced modified glycopeptides. The results demonstrated the successful incorporation of various 3,5-dihydroxyphenylglycine analogs. Mutasynthesis has shown to be applicable to the AB macrocycle to generate antibiotically active vancomycin-type derivatives, which are modified selectively at the residue of the 3,5-dihydroxyphenylglycine. Additionally, in comparison to chemical synthesis, mutasynthesis is much less laborious and therefore much more prolific.[22]

Another paper by Weist that uses mutasynthesis, "Fluorobalhimycin–A New Chapter in Glycopeptide Antibiotic Research," focuses on the development of fluorinated derivatives of the glycopeptide antibiotic balhimycin. Their aim was to elucidate the structure of all detected fluorobalhimycins, and to determine the configuration of the β-hydroxytyrosine stereoisomers that are accepted as substrates. A balhimycin-producing strain *Amycolatopsis mediterranei* was used to observe biosynthesis through gene disruption mutants. As a result, Weist et al. focused on two 3-chloro-β-hydroxytyrosine moieties, both of which were diastereomeric, using them as a target for the implementation of structural variations, as they are representative of a basic element of the tricyclic aglycon structure. The presence of a chlorine substituent in each of the two β-hydroxytyrosine moieties in a natural balhimycin played a significant role for enhancing antibiotic activity. From the wild-type strain *A. mediterranei*, a deletion null-mutant in the *bhp* gene was created called OP696, which is deficient in β-hydroxytyrosine biosynthesis, and as a result is unable to produce balhimycin. To proceed with mutasynthesis, OP696 was supplemented with several analogs of this amino acid. The results indicated that the β-hydroxytyrosine motif was essential for substrate acceptance in the different interactions of various glycopeptide biosynthesis enzymes, with peptide synthetases and oxygenases as the most directly affected ones. This sequence of interacting enzymes played a crucial role in differentiating between β-hydroxylated and non-β-hydroxylated tyrosines (D- and L-Tyr). Additionally, these enzymes also distinguish between tyrosine derivatives that possess the phenolic hydroxyl group at the 4-position of the arene and those that do not have this hydroxyl group. This complex cascade of enzymatic activity ensures precise modifications of the tyrosine molecules, impacting their biochemical properties and functions. However, substitutions of aromatic hydrogen atoms with fluorine in position 3 or 2 of the arene or even addition of a second fluorine substituent in position 5 of the arene is tolerated. In finding these effective fluorinated derivatives of glycopeptides, Weist et al. investigated resistance mechanisms to seek new approaches to overcome vancomycin-resistance problems.[23]

Another paper in which mutasynthesis is utilized to alter glycopeptides is "Altering glycopeptide antibiotic biosynthesis through mutasynthesis allows incorporation of fluorinated phenylglycine residues." In this article, nonribosomal protein synthetases are applied to engineer glycopeptide antibiotics. Nonribosomal protein synthetases are modular enzymes that catalyze synthesis of important peptide products from a variety of standard and non-proteinogenic amino acid substrates. Nonribosomal protein synthetases deliver amino acid and peptide intermediates, covalently bound to the pantetheine cofactor of a peptidyl carrier protein, to different catalytic domains where the nascent peptide chain is elongated, modified, and ultimately released. Within a single module are multiple catalytic domains that are responsible for incorporation of a single residue. After the amino acid is activated and covalently attached to an integrated carrier protein domain, the substrates and intermediates are delivered to neighboring catalytic domains for peptide bond formation or, in some modules, chemical modification.[24] In this work, an nonribosomal protein synthetase was utilized to incorporate non-proteinogenic amino acids, specifically to create glycopeptide antibiotic derivatives that have fluoridated phenylglycine residues. Modified phenylglycine residues were incorporated into nonribosomal protein synthetase biosynthesis pathways to control the structure of the glycopeptide antibiotics. Deletion of the native phenylglycine biosynthetic pathways resulted in the abolishment of production of the natural products since they provide these residues. Nonribosomal peptide derivatives can be created by supplementing amino acids called mutasynthones that, although structurally related, are unnatural, into the mutasynthesis strain. The mutasynthones were implemented to the culture of an *A. balhimycina* DSM5908 Δ*hpg* strain. The initial analysis of this unexpectedly demonstrated no production of a modified balhimycin peptide or biosynthetic precursors. However, 2-F-phenylglycine was accepted and successfully synthesized derivatives of novel fluorinated balhimycin. Mutasynthesis was also successful in the implementation for phenylglycine, 4-hydroxyphenylglycine, and

3,5-dihydroxyphenylglycine. Interestingly, the utilization of the nonribosomal protein synthetase in this mutasynthesis was limited due to poor acceptance of the modified substrates by the nonribosomal protein synthetase machinery. The phenol moiety in the 4-hydroxyphenylglycine residue was critical for the machinery to work and for the sequential cyclization activity of Oxy enzymes. This study illustrates the importance of maintaining key functional groups in order to successfully biosynthesize both the linear peptide by the nonribosomal protein synthetase and the subsequent cyclization by the Oxy enzymes and thereby engineer novel fluorinated glycopeptide antibiotics that are crosslinked and retain all other modifications including methylation, chlorination and glycosylation that are typically required to produce an active glycopeptide antibiotic.[25]

In comparing the effectiveness of these two methods shown in each paper, enzymatic glycosylation offers more control in alteration. Its addition of sugar moieties allows for more targeted refinements in the structure and, therefore, a function of the glycopeptide antibiotics. Additionally, enzymatic glycosylation is uniquely used for glycopeptides and glycopeptide antibiotics. On the other hand, mutasynthesis, which, of course, includes the addition of a precursor to the glycopeptide, allows for more possibilities and variations with glycopeptide antibiotics. While enzymatic glycosylation is highly precise and predictable, mutasynthesis provides a wider array of structural possibilities, making it a robust strategy for overcoming antibiotic resistance. Both methods are effective, but their strengths lie in different aspects of the modification process. However, although enzymatic glycosylation might be efficient for glycopeptide antibiotics, it unfortunately does not expand its use past strictly glycopeptides. Therefore, it would be unproductive with the β-lactam antibiotics previously mentioned. Additionally, although nonribosomal protein synthetase machinery is compatible with mutasynthesis, at times, lower incorporation efficiency has been demonstrated by some fluorinated analogs. Further improvement of nonribosomal protein synthetase machinery is needed to properly amalgamate the method with the machinery.

Conclusion and Discussion

Each technique discussed in the various papers on β-lactams and glycopeptides is generally effective, but serves different purposes, making them all suitable for different circumstances. A key example is enzymatic glycosylation, which is primarily used for glycopeptides, specifically glycopeptide antibiotics. While enzymatic glycosylation works well for glycopeptide antibiotics, it works well for *only* glycopeptide antibiotics, and other natural products require different combinatorial biosynthesis methods since enzymatic glycosylation is not effective for them. Techniques like precursor-directed biosynthesis, genetic engineering, and combinatorial biosynthesis each offer unique advantages for modifying natural products to create new antibiotics, with varying degrees of effectiveness and applications.

Precursor-directed biosynthesis, more specifically substrate-directed biosynthesis, and mutasynthesis, focuses on incorporating synthetic intermediates into existing glycopeptide pathways, which can lead to glycopeptides with altered activity and reduced resistance issues. By employing substrate-directed biosynthesis, researchers can direct the pathway toward desired products with specific mechanisms of action. This method can be quite predictable when the biosynthetic enzymes and pathways are well characterized. Although mutasynthesis, a combination of both precursor-directed biosynthesis and genetic engineering, is not always precise in its variation of antibiotics, it is very diverse in its alteration and has the potential to be utilized in a multitude of ways. Interestingly, although both can be considered precursor-directed biosynthesis, substrate-directed biosynthesis is much more precise in modifying natural products to create novel antibiotics. However, both techniques have some limitations: substrate-directed biosynthesis requires appropriate substrates that fit into the enzyme that is being reconstructed, and mutasynthesis is not always successful.

Genetic engineering provides a strong, succinct approach to mutate enzymes in order to improve their activity towards making novel antibiotics. Because it involves adjusting the structure of the enzyme that creates the antibiotic, genetic engineering might be considered a more complex technique; however, there is a wide range of uses for genetic engineering, creating a lot of diversity in its products. Genetic engineering offers high precision, allowing researchers to introduce specific changes to pathways that can yield antibiotics with known mechanisms of action. The homologous and heterologous expression of many natural product biosynthetic genes from divergent sources has been enabled by the advancements made in genetic engineering and has thereby resulted in the production of an abundant amount of enzymes from natural products by expressing their biosynthetic genes in other organisms.[26] As such, genetic engineering is crucial for the future of natural products, as it is extremely effective in honing in on certain aspects of an organism's DNA, isolating certain genes, and, therefore, encoding certain characteristics that result in an altered antibiotic.

Combinatorial biosynthesis, specifically for enzymatic glycosylation of glycopeptides, is highly effective, allowing the exploration of numerous structural variations to improve antibacterial properties, adding or altering sugar moieties, which in turn amplifies the solubility, stability, and binding affinity of glycopeptides, which can be particularly effective for improving the pharmacological properties of these antibiotics. It provides a lot of control to its appliers so that specific alterations can be made, unlike mutasynthesis. Although enzymatic glycosylation is limited to glycopeptides, combinatorial engineering can be used for a variety of other natural products. In general, combinatorial biosynthesis allows for the rapid generation of diverse analogs and can lead to new structures and activities. There is also some predictability in the types of compounds produced as known biosynthetic modules are combined.

Engineering the natural product pathways of nonribosomal protein synthetases is a frequently utilized application for the techniques described above. Due to their modularity and flexibility, they have become increasingly favored in the application of genetic engineering, combinatorial biosynthesis, and substrate-directed biosynthesis, particularly exemplified in the engineering of glycopeptides. Through metabolic engineering, hybrid nonribosomal protein synthetases can alter the peptide structure, targeted-gene disruption can alter the availability of precursors for nonribosomal protein synthetase utilization or remove unwanted moieties, and enzymes from other biosynthetic pathways can introduce new moieties.[27] Furthermore, the substrate tolerance of these pathways can be used to introduce nonnative amino acids to generate products with new peptide structures through precursor-directed biosynthesis. Nonribosomal peptide natural products include antibiotics like actinomycin, penicillin, cephalosporin, and vancomycin. Additionally, they also include other natural products, such as cytotoxics, for instance, bleomycin, and immunosuppressants, for instance, cyclosporine. Overall, they have played an essential part in medication for humans.[28,29] Nonribosomal protein synthetase engineering has a lot of potential for the future, as well, due to its applicability in so many natural products. However, it also has its flaws; it is challenging to utilize due to its complexity.

In general, studying the effectiveness of different methods of altering natural products to create novel antibiotics is significant in evolving biochemistry, particularly in the field of medicine, as antibiotics are so relevant. These altered antibiotics lead to the development of more effective medicine for humans that is more resistant to bacteria and is more specific in its medical support. Methods such as genetic engineering, precursor-directed biosynthesis, and combinatorial engineering are already being used to create novel antibiotics; for example, pristinamycin has been made by employing mutasynthesis to alter pristinamycin I based on the amino acid precursor phenylglycine.[30] Similarly, combinatorial biosynthesis has been applied to develop novel antibiotics related to daptomycin.[31] Understanding the variations between these different natural products' properties allows for antibiotic production to be quicker and more effective.

Acknowledegments

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