Rational Design of Macrolides by Virtual Screening of Combinatorial Libraries Generated through in Silico Manipulation of Polyketide Synthases

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Bacterial secondary metabolites display diverse biological activities, thus having potential as pharmacological agents. Although most of these compounds are discovered by random screening, it is possible to predict and re-design their structures based on the information on their biosynthetic pathways. Biosynthesis of macrolides, governed by modular polyketide synthases (PKS), obeys certain rules, which can be simulated in silico. PKS mode of action theoretically allows for a huge number of macrolides to be produced upon combinatorial manipulation. Since engineering of all possible PKS variants is practically unfeasible, we created Biogenerator software, which simulates manipulation of PKS and generates virtual libraries of macrolides. These libraries can be screened by computer-aided prediction of biological activities, as exemplified by analysis of erythromycin and macrolactin libraries. This approach allows rational selection of macrolides with desired biological activities and provides instructions regarding the composition of the PKS gene clusters necessary for microbial production of such molecules.

Introduction

Many biological species (bacteria, fungi, algae, sponges, plants, etc.) produce secondary metabolites with diverse biological activities, thus representing a rich source of potentially valuable pharmacological agents.[1,2] Currently, the majority of drugs derived from secondary metabolites were discovered by random screening of biological samples and subsequent rigorous testing. Examples of such compounds are amphotericin, erythromycin, cyclosporin A, paclitaxel, lovastatin, etc.[3]

Despite the utilization of high-throughput and ultra-high-throughput screening, major limitations of these approaches still exist and are related to (1) focusing on particular targets, which overlooks many useful kinds of biological activity, and (2) a rather low ratio of active to inactive molecules among the compounds tested experimentally. Various strategies and different computer-assisted approaches are proposed for library design to increase the chance of finding active hits and leads.[4−6] However, most of the existing target-based and ligand-based methods do not overcome the limitations described above, and this is particularly true for libraries of natural compounds.

In this paper we propose a general approach based on combination of the results achieved by new postgenomic technologies with methods of chemoinformatics. On the basis of today's knowledge on the biosynthesis of secondary metabolites, it is now possible to apply a rational approach based on in silico generation of secondary metabolites' structures in correspondence with the appropriate biosynthetic pathways. These combinatorial libraries can be further analyzed using computer-aided prediction of biological activities, physicochemical properties, drug-likeness, etc. Such analysis helps to select the most relevant compounds with respect to pharmacodynamic and pharmacokinetic properties.

This approach was tested using macrolides as an example, which are assembled biosynthetically by the modular polyketide synthase (PKS) enzymes. The macrolides biosynthesized by bacteria and fungi display a wide range of activities, including antibacterial, antifungal, immunosuppressive, antitumor, etc.[7] The PKS architecture and their mode of action (see the next section) theoretically allow for an enormous number of macrolides to be produced upon combinatorial manipulation of these enzymes, which cannot be engineered and tested experimentally. Under combinatorial manipulations we mean all possible perturbations of the PKS system via module and domain deletion/insertion as well as site-specific mutagenesis leading to either inactivation/reactivation of domains or changes in the domain's specificity toward a particular substrate. We developed a computer program Biogenerator that simulates in silico the action of modular PKS. The software generates combinatorial libraries of macrolides' structures for which computer-aided prediction of biological activities can be carried out. The results of the prediction can subsequently be used for selection of compounds with the required pharmacotherapeutic effects but without unwanted adverse and toxic actions.

In a case study for libraries consisted of erythromycin and macrolactin analogues it was shown that this approach provides reasonable results. In addition to the rational selection of macrolide molecules with the desired biological activities, the developed methods provide precise information on the composition of the appropriate PKS gene clusters required for biosynthesis of such molecules. Therefore, artificial PKS gene clusters for biosynthesis of these macrolides can be constructed in bacteria, allowing production and testing of the selected compounds.

Biosynthesis of Macrolides by Polyketide Synthases

The macrolactone rings of macrolides are formed via cyclization of polyketide chains assembled by the polyketide synthase (PKS) type I enzymes that perform repetitive decarboxylative condensations of carboxylic acids with an activated

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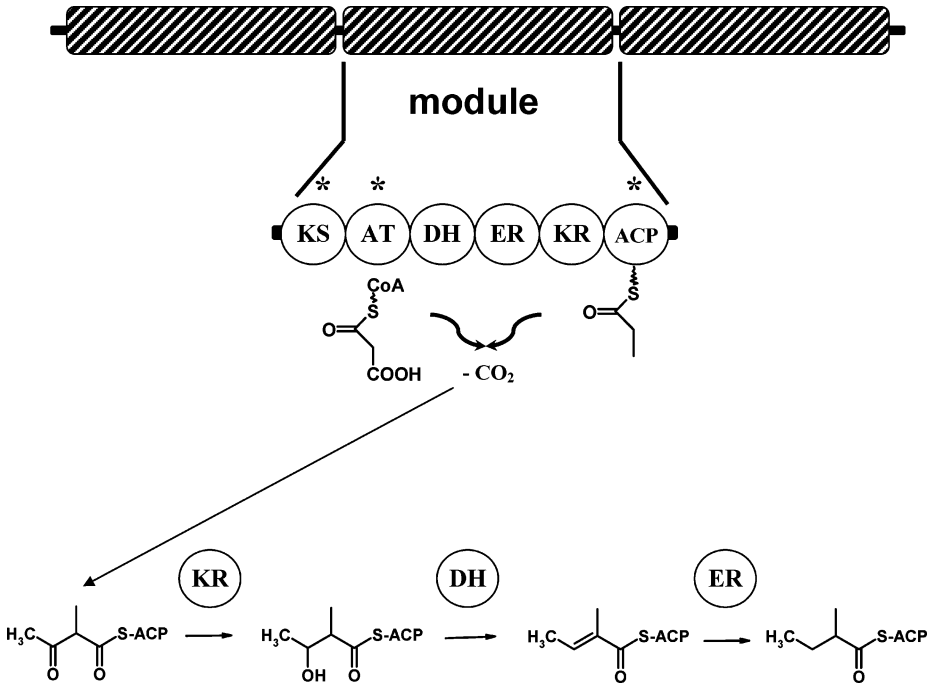


Figure 1. Organization and enzymatic reactions performed by the modular PKS during assembly of the polyketide chain, which is later cyclized to form a macrolactone ring. Enzymatic domains: KS, β-ketoacylsynthase; AT, acyltransferase; KR, ketoreductase; DH, dehydratase; ER, enoyl reductase; ACP, acyl carrier protein.

carboxylic acid starter unit.[8] PKS type I enzymes are composed of distinct modules, each of which is responsible for one condensation step. PKS modules contain a minimal set of β-ketoacylsynthase (KS), acyltransferase (AT), and acyl carrier protein (ACP) domains. The AT domain chooses a chain-building unit and transfers it to the ACP domain, where it is covalently tethered through a thioester bond to the phosphopantetheinyl arm. Claisen-type condensation with another unit held by the ACP on the adjacent module is catalyzed by the KS domain. The thioesterase (TE) domain located at the C-terminus of the last PKS module ensures termination of the polyketide chain synthesis, release, and cyclization of the product to form a macrolactone ring. Besides the core AT, KS, and ACP domains, PKS modules may contain up to three reductive domains possessing ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER) activities. The presence or absence of these reductive domains in a module determines the degree of reduction of the ketide unit introduced by the preceding module into the polyketide chain. Thus, the absence of these domains will lead to the appearance of a keto group on the macrolide molecule, while the presence of KR, KR+DH, and KR+DH+ER would yield hydroxyl, double bond, and saturated bond, respectively (Figure 1).

Such an intricate mechanism provides unique opportunities for changing the macrolide structures via manipulation of PKS domains and modules.[9] Several types of genetic manipulations of the modular PKS have been successfully accomplished over the past 10 years, resulting in production of new biologically active macrolides.[10] These manipulations included deletions of the PKS modules,[11] exchanges of AT domains between different PKSs and site-specific mutagenesis of ATs aimed at changing their specificities toward extender units,[12,13] and inactivation or/and addition of the reductive domains.[14] The apparent success of the manipulations described above suggests that PKS enzymes can tolerate a certain degree of structural rearrangements without losing their ability to assemble macrolides. It follows then that combination of all these manipulations might provide for the even greater chemical diversity of the macrolides synthesized by the genetically engineered PKS. Although there are still many limitations which might prevent production of desired macrolides, especially upon multiple manipulations of the naturally occurring PKS (e.g., no production or very low yields), several strategies might be employed to circumvent this problem. For example, one might choose to combine parts of several natural PKS systems with desired module and domain compositions in order to avoid introduction of multiple mutations/rearrangements in one system that might lead to loss of functionality.[15] Alternatively, recent technological advances allow the de novo synthesis of PKS genes or parts thereof, which can be combined and in most cases result in functional PKS systems yielding expected product.[16]

Most commonly the products of PKS, represented by macrolactone rings, are further modified by glycosylation, hydroxylation, methylation, etc., to give a final biologically active product. Therefore, it is important to consider the enzymes performing these reactions when manipulating the PKS gene clusters. Common post-PKS modification is glycosylation, i.e., attachment of a sugar moiety to the macrolactone ring by glycosyltransferases, which mostly perform O-glycosylations, attaching sugars through the free hydroxy groups on the aglycones.[17] Another type of enzyme frequently involved in post-PKS modification is represented by the P450 monooxygenases, the enzymes known to catalyze a wide range of reactions upon transfer of an oxygen atom from O_2 into various substrates. In the biosynthesis of macrolides the P450 monooxygenases are known to perform reactions such as hydroxylation,[10] epoxidation, and oxidation of a methyl group.[18]

Principles of Combinatorial Biosynthesis and Rationale behind the Computational Approach

The concept of combinatorial biosynthesis developed by Khosla and colleagues takes advantage of the guided PKS enzyme evolution to produce macrolide libraries.[19] Combinatorial biosynthesis was defined as "genetic engineering of the

biosynthetic pathways in such a way that they may be combinatorially reconstructed to produce libraries of novel small molecules that are appropriate for use in screening for new drugs".[19] However exciting the recent results on combinatorial biosynthesis seem at first glance, very few new macrolides can be produced in the laboratory.[10] It has also become evident that introduction of multiple substitutions in PKS is very laborious and often leads to substantially decreased yields of macrolides. It follows then that it is practically impossible to utilize the whole potential of most of the PKS systems described to date as the number of macrolides that they can theoretically produce is enormous. Calculation of the number of possible polyketides synthesized by a theoretical PKS is expertly described by Gonzalez-Lergier et al.[20] To exemplify the number of macrolides that can theoretically be produced upon manipulation of the largest PKS system studied, we made this calculation for the nystatin PKS, which contains 18 extender modules and utilizes both acetate and propionate extenders.[21] According to the equation proposed by Gonzalez-Lergier et al.,[20] the number of possible macrolides that can be produced via manipulation of the nystatin PKS is $2 \times 11 \times 18$. It shall, however, be kept in mind that introduction of post-PKS modifications such as glycosylation requiring a hydroxy group at certain positions of the ring reduces the number of variants. Nevertheless, it is evident that implementation of all these manipulations, as well as production, purification, and screening of all the resulting macrolides, is practically impossible.

Even if we consider the possibility that soon new technologies for fast and efficient manipulation of the PKS genes will become available, the problem with screening of biological activities for the products of engineered clusters will remain. A large number of biological activities have been described for macrolides, including antibacterial, antifungal, antitumor, antiviral, immunosuppressive, antiparasitic, etc.[7] Moreover, each of the biological activities is based on several mechanisms and targets that increase significantly the number of required assays. Considering this and a huge number of variants of the PKS-assembled molecules that can be theoretically produced, a substantial effort is required for in vitro screening. It is entirely possible that a very small change in the chemical structure can lead to a drastic alteration of biological activity. The latter relates not only to the loss of a specific activity intrinsic to the parent molecule, but also to the possibility of a completely new biological activity for the modified molecule. Taking into account the complexity of some assays and the vast number of different assays for biological activity that exist today, it seems unrealistic that any laboratory can perform a screening comprehensive enough to cover all possible activities that PKS-synthesized molecules can possess. Therefore, a considerable risk exists that some important activities will be overlooked. On the other hand, a computational approach to generation and virtual screening of macrolide combinatorial libraries can give an enormous advantage in selecting the structures with required biological activities and providing instructions for engineering of the corresponding PKS systems for their production.

General Methodology

Biogenerator Software. The completely rational approach to manipulating PKS, which allows modifications of the structure of macrolides in a predictable manner, is hindered by the fact that our current knowledge on structure−function relationships of antibiotics is insufficient. Thus, in most cases, manipulations of PKS and tailoring enzymes are done without any background information on how the resulting modification of the compound might affect its biological activity. It follows then that random manipulations of biosynthetic enzyme systems designed to introduce a great number of combinations of different modifications coupled to the high-throughput screening could yield new promising substances much more rapidly.

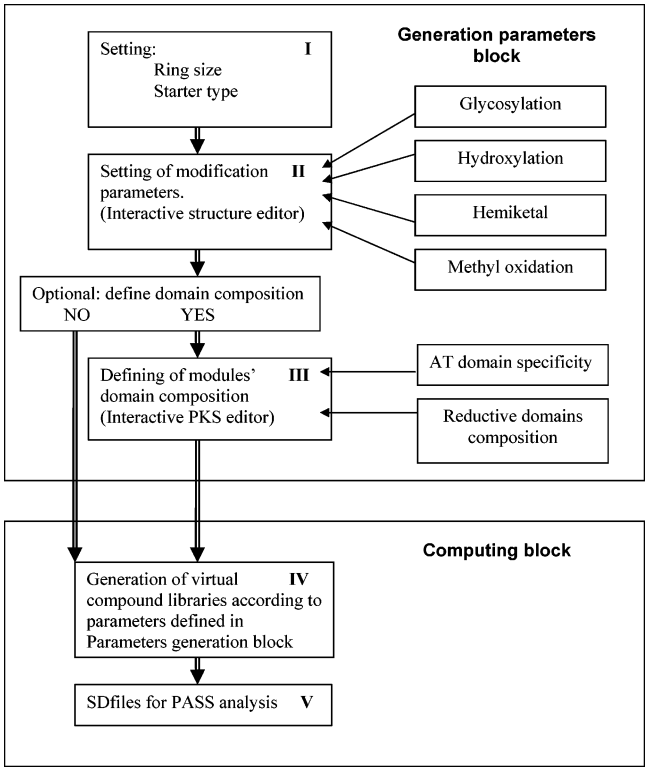


Figure 2. Flowchart showing the principles of Biogenerator software operation (see text for details).

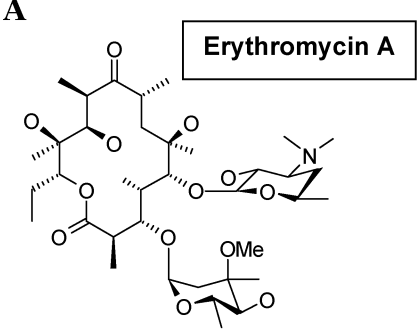
We developed the Biogenerator software, which performs in silico manipulation of theoretical PKS type I systems and is capable of creating virtual libraries of macrolide analogues. Optionally, the user can introduce post-PKS modifications at certain positions of the macrolactone rings. These libraries can be used for virtual screening based on computer-aided prediction of biological activity,[22−24] drug-likeness,[25,26] physicochemical properties,[27,28] and other characteristics related to the pharmacodynamics and pharmacokinetics of lead compounds.[29,30]

The Biogenerator software represents a bioinformatics tool for creation of virtual macrolide libraries based on in silico combinatorial manipulations of the modular PKS biosynthetic systems. The program output data are SD files, which contain molecular structures and PKS notations. The latter notation describes a domain composition and order of the modules in the hypothetical PKS enzyme system.

The flowchart in Figure 2 shows mode of operation for the Biogenerator software, which consists of two principle blocks: a generation parameters block and a computing block. The software allows a user to define the following generation parameters: **I** the **starter type** (acetate or propionate) and the **ring size** (up to 38 atoms) and thus the number of modules in theoretical PKS; **II** the following post-PKS **modification parameters** can be defined using graphic Interactive structure editor.

Glycosylation. A user is enabled to set post-PKS glycosylation sites at the putative hydroxyl groups, which could arise from PKS-directed reduction (i.e., only the KR domain in the given module is functional). The user can choose the type of

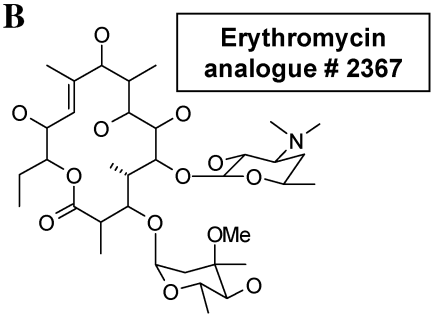
A



PASS ACTIVITY SPECTRUM at Pa > 0.7

Pa	Pi	
0.975	0.003	Antibacterial
0.970	**0.004**	**Protein synthesis inhibitor**
0.966	0.002	Protein 50S ribosomal subunit inhibitor
0.952	0.004	HERG channel antagonist
0.864	0.007	CYP3A1 substrate
0.854	**0.008**	**QT interval prolongation**
0.829	0.007	CYP3A substrate
0.811	0.006	Antidyskinetic
0.783	0.006	Selectin antagonist
0.766	0.011	CYP3A4 substrate
0.743	0.010	Antiprotozoal (Toxoplasma)
0.730	0.001	Testosterone antagonist

B



[Loading: Propionate]
[KS-ATmal--**-KR-ACP]**
[KS-ATmet-DH--**-KR-ACP]**
[KS-ATmet--**-KR-ACP]**
[KS-ATmal--**-KR-ACP]**
[KS-ATmal--**-KR-ACP]**
[KS-ATmet--**-KR-ACP]**

PASS ACTIVITY SPECTRUM at Pa > 0.7

Pa	Pi	
0.867	0.006	HERG channel antagonist
0.848	**0.005**	**Protein synthesis inhibitor**
0.800	0.004	Antimetastatic
0.798	0.004	Antibacterial
0.787	0.009	CYP3A1 substrate
0.758	0.021	Cholesterol synthesis inhibitor
0.746	0.010	Antidyskinetic
0.741	0.006	Selectin antagonist
0.743	0.012	CYP3A substrate
0.562	**0.056**	**QT interval prolongation**

Figure 3. Chemical structures and PASS-predicted biological activities for macrolides (A) erythromycin A and (B) erythromycin analogue 2367 from the virtual library created by Biogenerator (see text for details). Composition of type I PKS required for synthesis of analogue 2367 is provided by Biogenerator. ATmal, malonyl-specific acyltransferase domain; ATmet, methylmalonyl-specific acyltransferase domain.

attached sugar (currently D-desosamine, D-mycosamine, D-olivose, D-oliose, D-mycaminose, D-mycarose, L-daunosamine, L-oleandrose, L-cladinose) for each set position. All generated structures will contain chosen sugar(s) attached to the set positions. Consequently, all PKS notations will specify the corresponding modules as having only KR reductive activity required for appearance of a hydroxyl group chosen for sugar moiety attachment.

Hydroxylation. Post-PKS hydroxylation sites can be set at certain positions based on the user's knowledge on the specificity of particular enzymes (usually P450 monooxygenase) in the given biosynthetic system. Sites for hydroxylations do not overlap with the carbon atoms on the ring where hydroxy groups can appear as a result of PKS-catalyzed reduction by the KR domain.

Methyl Oxidation. In the case of polyene macrolides with a macrolactone ring size equal or over 22 atoms, a user can choose if a carboxy group will appear at a certain position. The carboxyl group arises from post-PKS oxidation of a propionate-derived methyl group on the macrolactone ring of polyene macrolides.[18] Therefore, if a user sets the parameter for a carboxy group to appear at a certain position, the propionate extender will automatically be chosen for the appropriate PKS module, as will be reflected in the PKS notation.

Hemiketal. A user can define the formation of a hemiketal, which can appear upon interaction of spatially proximal hydroxy and keto groups separated by three carbon atoms on the macrolactone ring. Setting the parameter for hemiketal formation will thereby restrict domain composition in two specific PKS modules.

Having set these modifications, a user has the option to start the generation process (**IV**) or define the domain specificity/composition of the PKS modules in which changes will not to be allowed during generation (**III**).

III the defining of **module's domain composition** using graphic Interactive PKS editor allows one to choose AT domain specificity (acetate or propionate) and reductive domain composition for each module.

When all these parameters are set up by the user, the software can generate virtual combinatorial libraries (**IV**). These output data are SD files. Each library contains both the chemical structures of the generated molecule and the PKS notation for the hypothetical enzyme system which could synthesize this compound. These PKS notations could be used as guidelines in development of recombinant bacterial cells producing selected compounds.

For example, a user can specify the chromophore and polyol regions that are necessary for pore-forming molecular complex

Table 1. PASS Prediction of Known Activity Types for 285 Erythromycin Analogues from the MDDR Database[a]

type of activity	*N*	±	Pa	Pi
acne therapy	7	0	0.738	0.005
antibacterial drugs	1	0	0.928	0.004
antibiotics	116	0	0.983	0.002
anti-helicobacter pylori agents	1	0	0.202	0.091
antimycobacterial agents	13	0	0.516	0.014
endometriosis therapy	7	7		
GnRH (LHRH) antagonists	18	0	0.587	0.002
macrolides	256	0	0.993	0.001
motilin receptor agonists	11	0	0.343	0.001
NF-(kappa)B activation inhibitors	1	1		
oncolytic drugs	11	0	0.427	0.095
prokinetic agents	11	0	0.858	0.002
prostate cancer therapy	7	0	0.584	0.003
quinolones	9	9		
treatment of protozoal diseases	1	0	0.565	0.012
treatment of tuberculosis	1	1		
sum	471	18		

[a] *N* = number of compounds with this type of activity. ± = number of compounds with experimentally confirmed activity which was not predicted by PASS. Pa = the maximal Pa value, which was predicted for each activity type. Pi = corresponding Pi value. (For each compound PASS estimates Pa and Pi = the probability to be active and inactive, respectively, for each activity type).

for polyene macrolides. This option is important if specific knowledge exists on the involvement of certain chemical groups in drug−target interactions.

Other starter and extender units as well as additional post-PKS modifications can be added to the Biogenerator's options if this will be necessary for generation of particular combinatorial libraries of macrolides.

Each block of Biogenerator software is written on a computer language corresponding to the task nature. All graphics are created on Tcl/Tk; all computational parts are the C++ programs.

Biological Activity Prediction. Since the presence of specific biological activity plays a key role in the discovery of new leads, computer-aided prediction of biological activity can be used as a first filter for selecting the most prospective compounds in virtual combinatorial libraries of macrolides produced by Biogenerator.

We used PASS software[22−24] as a tool for estimating biological activity spectra for the generated compounds because it can predict with reasonable accuracy more than 2000 pharmacological effects, mechanisms of action, and some specific adverse and toxic effects on the basis of structural formulas.[31] The mean prediction accuracy is about 85% according to the LOO CV estimation.[24] The PASS training set includes about 58 500 known biologically active substances (drugs, drug candidates, leads, and toxic compounds), including more than 1000 macrolides. These macrolides were reported to possess 241 biological activity types (see the Supporting Information). If the analyzed compound has a structure equivalent to the one in the training set, this structure, with all associated information about its biological activities, is "excluded" from the training set during the prediction.

It is necessary to stress that if the structure of the compound under prediction is equivalent to any structure from the training set, the last one, with all associated information about its biological activities, is excluded from the training set during the prediction. This approach provides more objective prediction results, and it was applied to all structures from MDDR used in the validation procedure.

PASS provides robust estimates of biological activity despite the incompleteness of data in the training set.[32] It was shown that based on PASS predictions a fraction of "actives" can be significantly increased in the selected subset.[33] New cognition enhancers[34] and anxiolytics[35] were identified in a virtual library of ∼5500 diverse compounds, and also some other successful applications were described earlier.[24] It is important to mention that PASS prediction of antineoplastic activity for secondary metabolites from marine sponges coincided with experimental data in almost 80% cases.[36]

In the predicted biological activity spectrum for a particular compound Pa and Pi are the estimates of probability to be active and inactive, respectively. Their values vary from 0.000 to 1.000. Only activities with Pa > T, where T is a user-defined threshold, are considered as possible for a particular compound. The result of prediction is presented as an SD file with the list of activities and appropriate Pa and Pi values sorted in descending order of the difference (Pa − Pi).

The higher the Pa value, the lower the probability of false positives in the set of compounds selected for biological testing. For example, if one selects for testing only compounds for which a particular activity is predicted with Pa ≥ 0.9%, the expected probability to find inactive compounds in the selected set is very low but about 90% of the active compounds are missed. If only compounds with Pa ≥ 0.8% are chosen, the probability of finding inactive compounds is also low but about 80% of active compounds are missed. By default, in PASS a Pa = Pi value is chosen as a threshold; therefore, all compounds with Pa > Pi are suggested to be active.

Another criterion for selection is the compounds' novelty. If the Pa value is high, sometimes one may find close analogues of known biologically active substances among the tested compounds. For example, at Pa > 0.7 the chance to find the activity in the experiment is high, but in some cases the compound may occur to be the close analogue of known pharmaceutical agents. At a Pa value between 0.5 and 0.7, the probability for the compound to be active in an experiment is less but the compound is not as similar to known pharmaceutical agents. If Pa for the compound is <0.5, this probability is even lower, but if the activity is confirmed, the substance might be regarded as a New Chemical Entity. On the basis of these criteria one may choose which biological activities have to be tested for the compounds on the basis of compromise between the novelty of pharmacological action and the risk to obtain a negative result in experimental testing.

A detailed description of the PASS algorithm is available elsewhere.[24] Activity spectra predicted by PASS can be used for further selection of compounds with the required biological activity spectra. For this purpose the PharmaExpert[37] computer program can be applied. This program provides a means for selection of compounds with required but without unwanted kinds of biological activity according to user-defined criteria. For example, a user can be interested in compounds that correspond to the query "5 hydroxytryptamine release stimulant predicted with probability more than 50% NOT convulsant". On the basis of PASS predictions for 880 compounds from the Prestwick Chemical Library[38] we identified 12 compounds that correspond to this query. PharmaExpert's application to selecting antihypertensive compounds with dual mechanisms of action was described previously.[23]

Software Availability. PASS can be used by the scientific community for free via the Internet from the web page.[39] Biogenerator will be available for licensing from Biosergen AS (Trondheim, Norway).

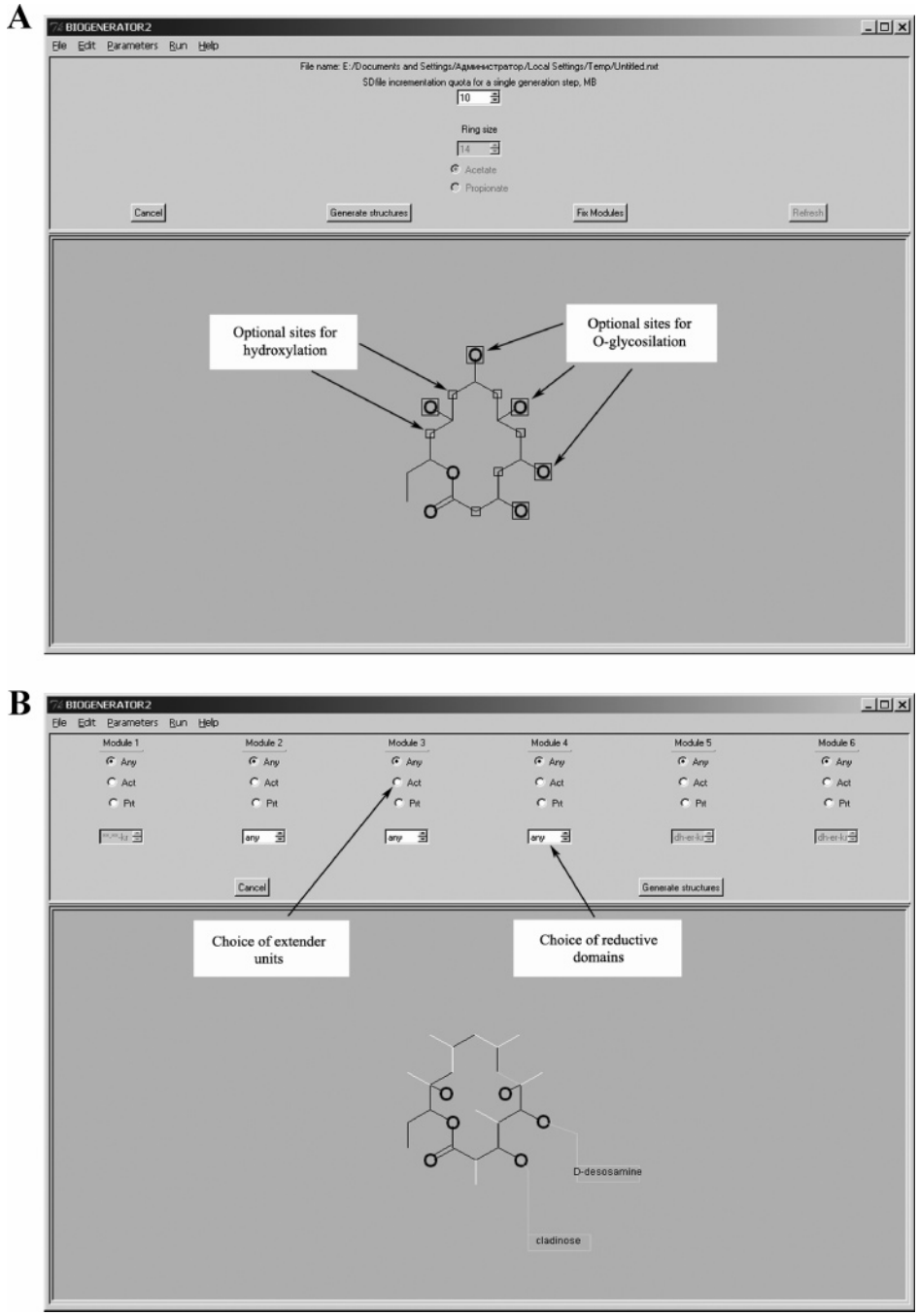


Figure 4. Biogenerator interface: (A) for setting up post-PKS modifications parameters; (B) for manual module composition setup.

Database Management System. For storage, visualization, and retrieval of virtual macrolide libraries we used DBMS ISIS Base (MDL Information Systems, Inc.).[40]

Results and Discussion

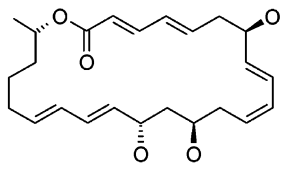
Generation and in Silico Analysis of the Erythromycin Analogues Virtual Library. To validate the applicability of the methodology described in the previous section, we generated and screened in silico a virtual library of erythromycin analogues. Erythromycin A (Figure 3A) is a well-known macrolide antibiotic with a 14-membered macrolactone ring which inhibits protein synthesis in bacteria through binding to the large ribosomal subunit close to the peptidyl transfer center.[41] The PKS gene cluster for erythromycin biosynthesis has been cloned and extensively studied,[42] and a substantial number of erythromycin analogues have been produced through both manipulation of the erythromycin PKS and chemical modifications.[7,10]

Prior to the analysis of the library created by Biogenerator, we performed a pilot test of PASS on the library of 285 erythromycin analogues with experimentally confirmed biological activities from the MDDR database.[40] The results of this analysis are presented in Table 1, which also shows the distribution of different types of activities within the library. The total number of experimentally confirmed activities (471) is larger than the number of compounds (285) due to the fact that many molecules in the library exhibit more than one type of activity. As is evident from the table, PASS failed to predict biological activities only in 18 of 471 cases, giving an accuracy of prediction of ca. 96%. The latter fact confirmed that the

Table 2. Statistical Distribution of Biological Activities (at Pa > 0.7) Assigned by PASS to the Library of 1060 Semisynthetic Erythromycin Analogues from MDDR Database and 3072 Erythromycin Analogues Created by the Biogenerator Software

predicted biological activity (Pa > 0.7)	MDDR (1060 structures)	Biogenerator (3072 structures)	refs[a]
HERG channel antagonist	1051	3072	43, 44
CYP3A1 substrate	639	3072	46, 47
protein synthesis inhibitor	527	3067	41
antibacterial	979	3017	41
antidyskinetic	465	2836	48, 49
CYP3A substrate	280	2261	45, 46
QT interval prolongation	466	1699	44
CYP3A5 substrate	37	1601	46, 47
selectin antagonist	130	1402	51
cholesterol synthesis inhibitor	6	1271	
protein 50S ribosomal subunit inhibitor	207	1206	41
CYP3A4 substrate	155	1041	46, 47
GDP-mannose 6-dehydrogenase inhibitor	108	440	50
antimetastatic		432	52
prokinetic	120	290	48, 49
gastrointestinal motility stimulant	97	190	48, 49
H^+ transporting two-sector ATPase inhibitor	3	167	
antineolpastic (lymphocytic leukemia)	7	153	52, 58, 59
antineolpastic (non-Hodgkin's lymphoma)	7	153	52, 58, 59
antineoplastic	1	115	52, 58, 59
CYP3A3 substrate	29	85	46, 47
testosterone antagonist	161	79	
CYP2C8 substrate	2	43	46, 47
flavanone 4-reductase inhibitor	4	35	
antiprotozoal (toxoplasma)	418	17	54
interferon antagonist	1	15	55
CYP2B6 substrate	2	11	46, 47
tumor necrosis factor alpha antagonist	14	7	56
membrane integrity antagonist	-	6	
potassium channel (voltage-sensitive) antagonist	17	4	
antimycoplasmal		3	57
immunosuppressant	1	3	53
CYP3A7 substrate	5	2	46, 47
fructan β-fructosidase inhibitor		1	
CYP2A substrate	2	1	46, 47
CYP2A6 substrate	2	1	46, 47
phosphatase inhibitor	15		
motilin receptor agonist	7		
microtubule formation stimulant	6		
interleukin antagonist	10		

[a] References providing relevant experimental evidence for a specific biological activity of structurally related macrolides.



Macrolactin A PKS:
[Loading: Acetate]
M1: [KS-ATmal--**-KR-ACP]**
M2: [KS-ATmal-DH-ER-KR-ACP]
M3: [KS-ATmal-DH--KR-ACP]**
M4: [KS-ATmal-DH--KR-ACP]**
M5: [KS-ATmal--**-KR-ACP]**
M6: [KS-ATmet--**-KR-ACP]**
M7: [KS-ATmet-DH--KR-ACP]**
M8: [KS-ATmet-DH--KR-ACP]**
M9: [KS-ATmet--**-KR-ACP]**
M10: [KS-ATmet-DH--KR-ACP]**
M11: [KS-ATmet-DH--KR-ACP]**

PASS ACTIVITY SPECTRUM at Pa > 0.7

Pa	Pi	
0.938	0.003	Cholesterol synthesis inhibitor
0.916	0.002	Antimetastatic
0.784	0.008	H+-transporting two-sector ATPase inhibitor
0.818	0.054	Dextranase inhibitor
0.737	0.028	Antineolpastic (lymphocytic leukemia)
0.737	0.028	Antineolpastic (non-Hodgkin's lymphoma)
0.759	0.059	Phosphatase inhibitor
0.711	0.012	Apoptosis agonist
0.709	0.096	Acylcarnitine hydrolase inhibitor
0.703	0.009	CYP2A11 substrate

Figure 5. Chemical structure of macrolactin A with deduced composition of PKS required for its biosynthesis and PASS-predicted biological activities (Pa > 0.7).

current version of PASS can be used for rather accurate prediction of biological activities during the screening of the virtual macrolide libraries.

Combinatorial biosynthesis utilizing erythromycin PKS gene cluster was modeled by Biogenerator using the following set of parameters (thus significantly limiting the size of the virtual library): propionate starter, 6 PKS extender modules, glycosylation with cladinose at C-3, glycosylation with desosamine at C-5, and hydroxylations at C-6 and C-12. Biogenerator interfaces that allow setting of the post-PKS modifications and

Table 3. Statistical Distribution of Biological Activities (at Pa > 0.7) Assigned by PASS to the Library of 1 048 576 Macrolactin A Analogues Created by the Biogenerator Software

predicted biological activity (Pa > 0.7)	no. of analogues	refs[a]
cholesterol synthesis inhibitor	1048 576	63
antimetastatic	1048 576	62
dextranase inhibitor	973 021	
antineolpastic (lymphocytic leukemia)	939 782	62
antineolpastic (non-Hodgkin's lymphoma)	939 782	62
P-benzoquinone reductase (NADPH) inhibitor	693 729	
phosphatase inhibitor	628 618	
acylcarnitine hydrolase inhibitor	613 330	
H^+ transporting two-sector ATPase inhibitor	429 223	
trans-pentaprenyltransferase inhibitor	300 178	
CYP2B5 substrate	270 376	
flavanone 4-reductase inhibitor	258 170	
CYP2A11 substrate	255 784	
CYP2A2 substrate	205 430	
CYP2A4 substrate	200 104	
toxic	183 142	
prostaglandin-E2 9-reductase inhibitor	132 417	
immunosuppressant	110 082	
apoptosis agonist	107 181	62
antiseborrheic	92 943	
Bcl2 antagonist	75 703	62
oxidoreductase inhibitor	42 321	
teratogen	41 891	
carbonyl reductase (NADPH) inhibitor	20 388	
CYP2A1 substrate	19 710	
glyoxylate oxidase inhibitor	17 718	
prolyl aminopeptidase inhibitor	9 503	
membrane integrity antagonist	9 476	
atherosclerosis treatment	7 540	
interferon antagonist	6 038	
membrane permeability enhancer	5 701	
CYP3A5 substrate	5 178	
HERG channel antagonist	4 268	
cardiovascular analeptic	2 058	
alcohol oxidase inhibitor	1 078	
licheninase inhibitor	1 031	
glycerol-1-phosphatase inhibitor	1 023	
hypertensive, ophthalmic	1 008	
microtubule stabilizator	236	
lactaldehyde reductase inhibitor	233	
pectin lyase inhibitor	56	
scytalone dehydratase inhibitor	55	
CYP2C8 substrate	35	
hypolipemic	22	
NADPH peroxidase inhibitor	4	
trans-cinnamate 4-monooxygenase inhibitor	3	
CYP2B6 substrate	1	
levanase inhibitor	1	

[a] References providing relevant experimental evidence for a specific biological activity of structurally related macrolides.

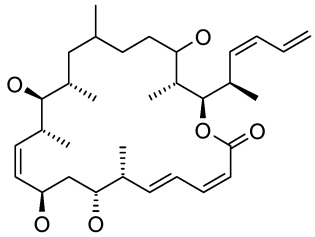
PKS module compositions are shown in Figure 4A and B, respectively. All extension PKS module compositions were left variable with respect to the extender unit (acetate or propionate) and reductive domains. It should be noted that the stereochemistry at the possible chiral centers was left out in the current version of the program since the MNA descriptors used in PASS currently do not discriminate the stereochemistry.

The virtual erythromycin analogue library created by Biogenerator based on the set of parameters described above contained 3072 structures. The library was analyzed by the PASS software, predicting biological activity spectra for these molecules at a Pa threshold over 0.7 (predicted probability of being active over 70%). The PASS results were analyzed by the PharmaExpert software, which allows statistical analysis as well as screening for specific biological activities assigned by PASS to the chemical structures in the library. In parallel, a virtual reference library of 1060 semisynthetic erythromycin analogues from the MDDR database[40] was analyzed in a similar manner. It shall be noted that the latter library is considerably more structurally diverse due to addition of different chemical groups to the macrolactone ring of erythromycin by synthetic chemistry. The latter most probably accounts for the wider range of biological activities predicted by PASS for the reference library compared to the Biogenerator library. The overview of the results and distribution of different activities in the two libraries is presented in Table 2.

The results of prediction identify several kinds of activity that have been experimentally discovered over recent years for erythromycin and related macrolides and do not seem to be related to their antibacterial activity. In particular, PASS predicted such experimentally confirmed activities as HERG channel antagonist and QT interval prolongation,[43−45] CYP3A family substrate,[46,47] antidyskinetic,[48,49] GDP-mannose 6-dehydrogenase inhibitor,[50] selectin antagonist,[51] antimetastatic,[52] immunosuppressant,[53] antiprotozoal,[54] interferon antagonist,[55] tumor necrosis factor alpha antagonist,[56] and antimycoplasmal.[57] Remarkably, PASS predicted antineoplastic activity for 140 erythromycin analogues in this library. In this respect, it is noteworthy that recent studies on a chemically modified erythromycin analogue, roxithromycin, revealed its angiogenesis inhibitor activity in vivo.[58] Another erythromycin derivative, clarithromycin, was shown to induce apoptosis in murine B cell lymphoma cells.[59]

In certain cases, such as membrane integrity antagonist activity predicted for six members of the Biogenerator erythromycin analogue library, no direct experimental evidence could be found in the literature. However, this type of activity can be rationalized from a recent observation on the erythromycin analogue azithromycin, which was shown to enhance membrane fluidity by eroding the DPPC and DPPE gel domains.[60] No experimental data was found for the PASS-predicted activities such as cholesterol synthesis inhibitor, H^+ transporting two-sector ATPase inhibitor, testosterone antagonist, and flavanone 4-reductase inhibitor. Since such activities were predicted only for certain members of the libraries, it may be that these activities are specific for these compounds and have never been assayed for known erythromycin analogues.

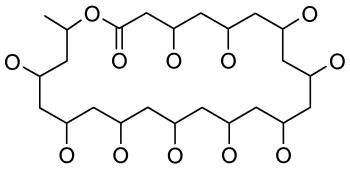
To demonstrate the output that our approach can provide, we performed virtual screening of the Biogenerator erythromycin analogue library using specific undesired activity "QT interval prolongation". Erythromycin has been shown to cause prolongation of cardiac muscle repolarization (QT interval), which may lead to ventricular arrythmias.[45] Most likely, this property of erythromycin can be attributed to the desosamine moiety, which contains a nitrogen atom linked to three carbons, and thus can represent a "QT pharmacophore" suggested by Cavalli et al.[61] Screening of the 3072-membered erythromycin library was performed with Pa < 0.6 for the "QT interval prolongation" activity and Pa > 0.8 for "Protein synthesis inhibitor" activity thresholds. The virtual screening of the Biogenerator library with these parameters yielded 26 erythromycin analogues. One analogue, 2367 in the virtual library, having the lowest predicted Pa ratio for the two activities used in the screen, is presented in Figure 3B. This figure also depicts the composition of PKS that will be required for biosynthesis of this molecule, which is automatically provided by Biogenerator and an activity spectrum predicted by PASS. All this information could be retrieved using ISIS Base software[40] from the SD file saved after screening the PASS-generated activity spectra prediction file with PharmaExpert. On the basis of these calculations, erythromycin analogue 2367, which can be produced via manipulation of



Dictyostatin-1

PASS ACTIVITY SPECTRUM at Pa > 0.7

Pa	Pi	
0.922	0.006	Phosphatase inhibitor
0.895	0.005	Cholesterol synthesis inhibitor
0.827	0.006	H+transporting two -sector ATPase inhibitor
0.803	0.004	Antimetastatic
0.784	**0.003**	**Microtubule stabilizator**
0.744	**0.005**	**Microtubule formation stimulant**
0.727	0.008	Immunosuppressant
0.723	0.004	Membrane Permeability enhancer
0.762	0.044	Beta-adrenergic-receptor kinase inhibitor
0.715	0.011	CYP2A substrate



Marolactin analogue # 49526

PASS ACTIVITY SPECTRUM at Pa > 0.7

Pa	Pi	
0.951	0.003	Cholesterol synthesis inhibitor
0.929	0.004	Antineolpastic (lymphocytic leukemia)
0.929	0.004	Antineolpastic (non -Hodgkin's lymphoma)
0.910	0.016	Dextranase inhibitor
0.863	0.003	Antimetastatic
0.845	0.040	Acylcarnitine hydrolase Inhibitor
0.778	0.052	Flavanone 4-reductase inhibitor
0.773	0.015	Membrane integrity Antagonist
0.771	0.054	Phosphatase inhibitor
0.762	0.007	Apoptosis agonist
0.759	0.006	Pectin lyase inhibitor
0.749	**0.003**	**Microtubule stabilizator**
0.755	0.012	Bcl2 antagonist
0.745	0.007	CYP3A5 substrate
0.747	0.016	Glycerol-1-phosphatase inhibitor
0.736	0.010	Atherosclerosis treatment

Figure 6. Chemical structures and PASS-predicted spectra of biological activities (Pa > 0.7) for distyostatin-1 (A) and macrolactin analogue 49526 from the Biogenerator virtual library (B).

erythromycin PKS, might be a safer (compared to erythromycin A) antibiotic for patients predisposed to QT interval prolongation.

Generation and in Silico Analysis of the Macrolactin A Combinatorial Library. Macrolactins are 24-membered macrolides produced by unidentified marine bacterium, Actinomadura sp., and Bacillus sp., which exhibit both antibacterial and antitumor activities in vitro.[7] In addition, macrolactin A (Figure 5) was shown to inhibit mammalian herpes simplex virus and HIV replication.[62] Recently, it was shown that macrolactin A also efficiently inhibits squalene synthase[63] and thus has potential as an inhibitor of cholesterol biosynthesis. Although cloning of the macrolactin A biosynthetic genes has not been reported, its chemical structure suggests that it is assembled by a modular PKS system. Considering this, we generated a combinatorial library of macrolactin A using the Biogenerator software. The only restriction applied prior to library generation was the specificity of the modules toward acyl-CoA starter and extenders, which was set for acetate. The resulting virtual library consisting of 1 048 576 structures was analyzed by the PASS/PharmaExpert software. The statistical distribution of the predicted biological activities among the library members is presented in Table 3. It is important to note here that macrolactin A was not included in the training set of PASS, and therefore, this compound and its analogues were completely "unknown" to the software, even though PASS was able to predict biological activities for macrolactin A, which have been confirmed experimentally (Figure 5, Table 3).

As is evident from Table 3, there is a good correlation between several types of predicted activities with experimental data available for macrolactins. In particular, PASS predicted such confirmed activities as cholesterol synthesis inhibitor,[63] antineoplastic, and apoptosis agonist.[62] The antibacterial and antiviral activities confirmed for macrolactins by Gustafson et al.[62] were not directly predicted by PASS, presumably due to the specific mechanisms of action of these macrolides, which so far have not been described for these two types of activity. However, based on the PASS prediction, we speculate that the

antibacterial activity of macrolactins could be due to inhibition of the H^+-transporting two-sector ATPase, which is essential for viability of bacterial cells.[64,65] Antiviral activity of macrolactins could probably be attributed to the phosphatase inhibitor activity predicted by PASS. It has been reported that certain protein phosphatases are involved in regulation of HIV transcription.[66,67]

A wide range of other biological activities, for which no experimental evidence could be identified, was predicted by PASS for certain members of the macrolactin library. Those medically relevant and, from our point of view, worth mentioning are such activities as antimetastatic, acylcarnitine hydrolase inhibitor, antiseborrheic, cardiovascular analeptic, and microtubule stabilizator. Macrolactins structurally resemble dictyostatin-1, a 22-membered macrolide isolated from marine sponge and shown to have tubulin stabilizing activity.[68] Stabilization of a microtubule is a mechanism of action of several anitumor agents, such as taxol, epothilone, and discodermolide,[69] and thus can be considered an important property in searching for new anticancer drugs. We analyzed the structure of dictyostatin-1 using PASS, and the list of predicted biological activities for this compound at Pa > 0.7 is presented, along with the structure, in Figure 6A. Among the members of the macrolactin analogues virtual library created by Biogenerator, 236 compounds were predicted by PASS to have microtubule stabilizing activity with Pa > 0.7. We further screened a sublibrary of the abovementioned 236 macrolactin analogues in order to identify compounds with the highest Pa value for the microtubule stabilizing activity. Macrolactin analogue 49 526 was picked during this screen as having the best Pa score. Its structure along with the PASS-predicted activity spectrum is presented in Figure 6B. The composition of PKS modules retrieved from the Biogenerator SD file and corresponding to the structure of analogue 49 526 was in complete accordance with the results of manual prediction of hypothetical PKS for this analogue (data not shown).

Conclusion

We designed and implemented in silico a new approach to generation and screening of virtual macrolide libraries that can lead to discovery of new drug-like activities for these molecules. On the basis of the output of this software package, molecular biologists will be able to design and construct artificial PKS gene clusters for production of the most interesting molecules in bacteria. Validation of this approach performed on thousands of macrolides known from the literature has demonstrated the reasonable accuracy of computer prediction. Experimental testing on a few selected examples is currently under way.

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Supporting Information Available: List of activity types revealed by collected macrolides. This material is available free of charge via the Internet at http://pubs.acs.org.

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