COMBINATORIAL ENZYMOLOGY. SYNTHESIS OF NOVEL BETALACTAM LIBRARIES

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The methodology for the discovery of new biologically active betalactams is proposed. The one of the two ways proposed is *specific modification*, which is peculiar to any betalactam structure and involves introduction of substitutes changing particular physico-chemical properties of the natural or synthetic analogous.
The *principle of similarity* is discussed as an alternative of the specific modification in the design of new biologically active compounds. The distinctive feature of this approach is usage as building blocks substitutes favourable for the well-known in medical practice betalactam antibiotics and their "implantation" into other (new) betalactam structures. The paths of new betalactam synthesis, including the methods of enzyme engineering are considered. The possibility of usage of enzyme engineering processes for production of not only new individual betalactams— *hits*, but also for synthesis of the complex betalactams—leads, is shown.
More than 4500 new penicillins, cephalosporins and monobactams are constructed with accordance of the principle of similarity. More than halves of them can be produced by enzymatic synthesis or combination of chemical and enzymatic synthesis. The constructed compounds are enumerated in the tables, the request for the electronic version of which can be sent to the address: davidnys@writeme.com.

Introduction

Last years the new biologically active substance discovery is in a great demand. The key approach to such discovery is the combinatorial chemistry, by the methods of which collections of new compounds are constructed and proposed to the pharmaceutical companies for total screening. The amount of compounds in collections and number of collections frequently exceed the possibilities of high-throughput screening (HTS). So, there is a problem of design of the virtual compound libraries, which are made one or another feature representing sampling of molecules from the larger library. The different approaches to design of such virtual libraries are depicted [1].

The long-time experience in the field of enzymatic synthesis and transformation of antibiotics has allowed us to engage in design of new betalactam libraries. The analysis of literature data on the mechanism of antibiotic action and pathogen resistance as well as on the interrelation between the structure and biological activity of antibiotics is a basis of the methodology of new biologically active betalactam design [2]. Availability of the enzymes used in synthesis and transformation of betalactam antibiotics, and comprehension of the mechanism of their action allow to use the methods of enzyme engineering for production of the large number of betalactams from the virtual library, designed by us. The advantages of an enzymatic way in comparison with traditional chemical synthesis, connected with possibilities of reaction proceeding in mild conditions, make it possible to offer the combinatorial enzymology as an alternative of combinatorial chemistry. The use of the enzyme engineering processes for a new betalactam production is interesting also from the point of extension of a spectrum of practical implementation of technological enzymes for semisynthetic betalactam antibiotic production.

The main goal of the present work is to study the possibility of use of enzyme engineering methods for synthesis of new betalactam compounds.

Methods

Materials and Reagents

Biocatalysts. The immobilized penicillinamidase from *Escherichia coli* (strain NAT-99 from NRCA collection), immobilized synthetase of cephalosporin-acids from *E. coli* (strain 1787 from NRCA collection) and immobilized aminobetalactamsynthetase from *Xanthomonas rubrilineans* (strain VKM-629 [3]) were used as a biocatalysts for synthesis of new betalactams. The cultivation and enzyme isolation for both penicillin G amidase and synthetases of cephalosporin-acids from *E. coli* were carried out as described in [4], for aminobetalactamsynthetase from *X. rubrilineans* in [5]. The enzymes were precipitated from the cell-free extract, modified with glutaraldehyde and entrapped in polyacrylamide gel according to [6].

Key amino acids. 6-aminopenicillanic acid (6-APA), 7-aminodesacetoxicephalosporanic (7-ADCA) and 7-aminocephalosporanic acid (7-ACA) are the commercial products of Russia. 7-amino-3-methylmercaptothiadiazolylcephalosporanic acid (MMTD-7-ACA) is produced by Biochemie (Austria).

7-amino-3-chlorcephalosporanic acid (7-ACCA) is produced by Ranbaxi (India). 7-amino-3-vinylcephalosporanic acid (7-AVCA) and 7-amino-3-methylmercapto-(1-methyl-

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tetrazolyl)-cephalosporanic acid (TET-7-ACA) are received from Re Yon Farmaceutical Co. (Korea).

Acylating agents. The synthesized in NRCA methyl esters of carbonic acids, namely, phenylacetic (PAA), tetrazolylacetic (TzAA), and amino acids, D-penylglicine (DPG) and p-hydroxy-D-phenylglicine (DHPG), were used as acylating agents.

Synthesis of New Betalactams

Experiments on synthesis of new betalactams were carried out in glass reactor supplied with jacket for heating by hot water, paddle stirrer and pH-state system. A solution of initial reagents (30–50 mm of key amino acid, 2–2.5-fold excess of acylationg agent) in 0.1 M phosphate-ammonium buffer pH 6.0–6.5 is loaded in a reactor, an aliquot of the corresponding immobilized biocatalyst (5–7% weight on volume) is added, and reaction mixture is agitated at temperature 20–30 °C and pH 6.0–6.5 within 40–70 min. An immobilized biocatalyst is filtered out, a reaction mixture analysed by HPLC. The Waters Associates Inc. (USA) chromatograph was used. Sylasorb C18 with the particle size of 4.5 μm in diameter was applied as a stationary phase in a stainless steel column (250×4.0 mm). The rate of mobile phase flow is 1 ml/min. The mobile phases were a mixtures of methanol and phosphate-ammonia buffer in different ratio.

Results and Discussion

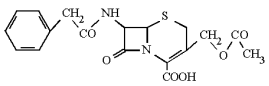
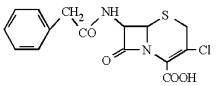
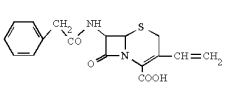
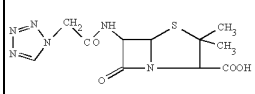
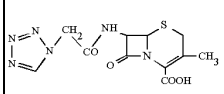
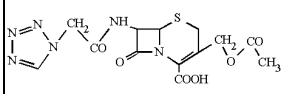
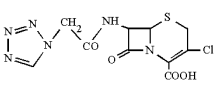
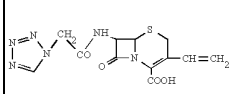
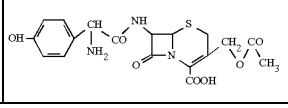
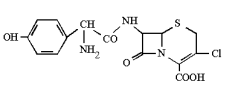
There are at least two ways for the design of new biologically active betalactams: the *specific modification* and/or combinatorial chemistry based on the "implantation" of the substitutes favourable for well-known in medical practice betalactam antibiotics into other structures. The first way is based on the account of the interrelation between the structure and biological activity of betalactams as well as on the comprehension of the role and physico-chemical properties of substitutes, introducing into natural or synthetic analogous. The specific modification requires the large deepness of the research and/or huge volume of experimental studies for every compound. The principles of the specific modification of biologically active compounds and the examples of the use of them for production of the high efficient betalactam antibiotics are depicted [7]. The volume and deepness of the physico-chemical studies required for attaining the aim are also specified in the publication [7].

The second approach to a new betalactam design is based on the *principle of similarity*, i. e., on the use of different combinations of acylating agents and key amino acids of known drugs as building blocks in construction of new structures. There are numerous examples in the literature illustrating the expediency of such an approach. First of all, close or identical structural modification of the side chain of the penicillin, cephalosporin and monobactam structures, as well as modifications by C (2) in the penem and carbapenem structures should be mentioned. In the latest heteryl, heterylmethyl and heterylthiomethyl systems known as constituents of highly active cephalosporin are used. The structures of known antibiotics and other betalactam compounds are depicted in [7].

It should be emphasized that other pharmacological properties of the compounds constructed according to the principle of similarity besides antibacterial activity can be revealed in total screening of new biologically active betalactams. The extension of spectrum of pharmacological activity of new compounds is connected with introduction of structural fragments, which are responsible for definite biological activity types, to betalactams. The following modification of the constructed compounds for increase of pharmacological effect can be performed if needed. In favour of

Table 1

Enzymatic synthesis of new betalactams

Acylating agents	Key compounds				
	6-APA	7-ADCA	7-ACA	7-ACCA	7-AVCA
Methyl ester (ME) of PAA	PenG	Ceph G			
ME TzAA					
ME DPG	Ampicillin	Cephalexin	Cephaloglicin	Cefaclor	—
ME DHPG	Amoxicillin	Cephadroxil			—

Notice. The structures of synthesized new betalactams are presented. The known antibiotics produced by enzymatic synthesis from corresponding compounds are named.

such an approach is, for example, the fact that some compounds whose structural fragments are used in construction of the penicillin side chain have not only marked antibacterial activity but also other biological activities such as vasodilative, non-specific anti-inflammatory, immunostimulating and others.

We used the principle of similarity to design new betalactam compounds. The compounds constructed are enumerated in electronic tables E.1–E.4*. The virtual library, which contains the betalactam structures on the base of penams, cephems and monobactams, is most widely presented. These compounds are produced by condensation of amino group of one of the further mentioned key amino acids with carboxylic group of carbonic acids, used in synthesis of semisynthetic penicillins, cephalosporins and monobactams, well-known in clinical practice (electronic table E.1). 6-APA, 7-ADCA, 7-ACA, and its 3-substituted analogous, 7-amino-7-methoxycephalosporanic acid and its 3-substituted analogous, 3-aminonocardic acid and others—60 compounds at all, were used as key amino acids. 85 carbonic acids were used as a source of acyl moieties. They are the following: derivatives of the α-disubstituted acetic acid, N-substituted-α-aminoacetic acid, α-iminoacetic acid and its analogous. The substitutes at C (3) position of key amino acids on the base of cephems as well as acyl fragments of molecules are taken from the highly active cephalosporins described in publication [7].

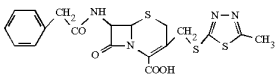
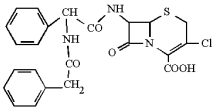
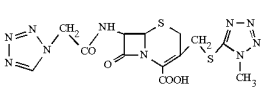
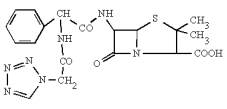
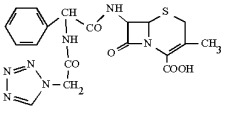
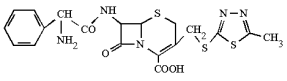
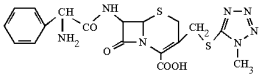
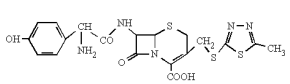
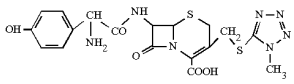
More than 4500 virtual structures have been constructed by coupling of before mentioned key amino acids and acylating agents. The number of new betalactam structures can be increased markedly by use of different coupling of substitutes in key amino acids and acylating agents enumerated in table E.1. The total number of such structures, estimated according to following equation for every structural type—Q_i, is about 150 000. About 1500 compounds from them are penams, 100 000—cephems and 48 000—monobactams:

$$Q_i = k_n\, a_n, \tag{1}$$

Here $k_n = k_1 k_2 \dots k_n$ and $a_n = a_1 a_2 \dots a_n$, where k is number of key amino acids used, a is number of acylating agents, and n is number of variable substitutes in structure of key amino acid or acylating agent.

More than halves of designed compounds can be produced by enzymatic synthesis. Enzymatic method of compound production is an alternative to the chemical one and has some advantages because it allows to proceed reactions in mild conditions (aqueous media, room temperatures, neutral pH), which are especially important for the betalactams—the labile compounds. The mechanism of action of enzymes—peptidohydrolases, used in the synthesis and transformation of betalactam antibiotics, was studied by us. The biocatalysts on the base of immobilized enzymes and/or cells as well as the processes for enzymatic synthesis of variety of betalactam antibiotics were developed [8–10]. The new individual betalactam compounds—so called *hits*, can be synthesized with using of immobilized penicillin amidase from *E. coli*, cephalosporin-acid synthetase from *E. coli* and aminobetalactam-synthetase from *X. rubrilineans*, which have the high substrate specificity in relation to derivatives of PAA, TzAA and DPG respectively. These hits are the penicillins and betalactam-acids—cephalosporins and monobactams first of all, as well the aminopenicillins, aminocephalosporins and their analogous with substituents in amino group of side chain—ureidopenicillins and ureidocephalosporins.

* The request for the electronic version of table can be sent to the address: davidnys@writeme.com.

Key compounds				
MMTD-7-ACA	TET-7-ACA	Ampicillin	Cephalexin	Cefaclor
	—	—	—	
Cefazolin				—
		—	—	—
		—	—	—

Key compounds

The above mentioned enzymes can be used also for the synthesis of compounds which content in acyl moiety the residue of other acids with the structure closed to the one of aforementioned acids. The compounds can be produced by enzymatic synthesis are marked in electronic table E.1. The possibility of enzymatic synthesis use for hit production was demonstrated experimentally.

The new betalactams produced by method of acyl transfer synthesis catalyzed by one of the indicated enzymes with acylenzyme intermediate formation mechanism of action are presented in Table 1. The new penicillins and cephalosporin-acids were synthesized by acylation of amino groups of well-known key amino acids (6-APA, 7-ADCA, 7-ACCA, 7-AVCA, 7-ACA, MMTD-7-ACA, and TET-7-ACA) as well as amino containing antibiotics (ampicillin, cephalexin and cefaclor) by methyl esters of PAA and/or TzAA acid with using of immobilized penicillin amidase or cephalosporin-acid synthetase from *E. coli*, correspondingly.

The new aminocephalosporins were produced with using 7-ACCA, 7-ACA, MMTD-7-ACA, and Tet-7-ACA as key amino acids, methyl esters of DPG and/or DHPG as acylating agents and immobilized aminobetalactamsynthetase from *X. rubrilineans* as biocatalyst. 19 new compounds were produced. The HPLC analysis was used to analyze the reaction mixture. The appearing of new peak on the chromatogram showed the new compound formation. The quantity of synthesized betalactam was estimated by key compound consumption. The level of key compound transformation achieved from 30 to 75%. Reaction mixture after enzymatic synthesis contents, besides a new product, the unreacted initial reagents and by-product—carbonic acid (PAA, TzAA, DPG, and DHPG in our cases), which have no biological activity. So, it is enough to lyophilise reaction mixture for the biological activity testing. If it need, the synthesized betalactam compound can be precipitated by changing pH, dielectric permeability of solution or by use of specific precipitator, i. e., one of the methods used for separation of components of betalactam antibiotic enzymatic synthesis [8].

As it was shown before the peptidohydrolases, used in synthesis and transformation of betalactam antibiotics, have a broad substrate specificity in relation to derivatives of aforementioned acids [11]. Due to this phenomenon as well as accounting that biocatalytic reaction mixtures are the model systems practically, not only *hits* but also *leads*—compound compositions—can be produced by the enzymatic synthesis successfully. The possibility of synthesis of a new betalactam compositions on the base of common acylating agent and different key amino acids was demonstrated by us on the model of synthesis of known antibiotic composition.

The composition of aminobetalactams—ampicillin, cephalexin, and cefaclor, was produced by acylation of the mixture of taken in equal concentrations (about 50 mM) three amino acids—6-APA, 7-ADCA, and 7-ACCA, by methyl ester of DPG, which concentration 2 times exceeds the sum of key amino acids, with using of aminobetalactamsynthetase from *X. rubrilineans*. The HPLC analysis of reaction mixture revealed the three aminobetalactams

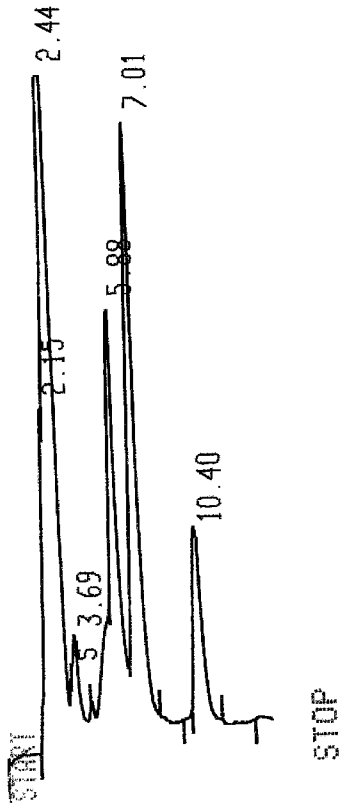


Fig. 1. The chromatogram of reaction mixture produced by acylation of the mixture of 6-APA, 7-ADCA, and 7-ACCA (Rt 2.1–2.3 min) by methyl ester of DPG (Rt 3.69 min) in presence of aminobetalactamsynthetase from *X. rubrilineans*. The experiment conditions: the initial sum concentration of key amino acids in 0.1 M phosphate-ammonia buffer pH 6.5 is 150 mM, the concentration of methyl ester of DPG is 375 mM; temperature is 30 °C; pH 6.1–6.2; the time of reaction is 70 min; the mobile phase for HPLC analysis is mixture of 27% of methanol with 73% of 0.05 M phosphate-ammonia buffer pH 2.05. The retention time of enzymatic synthesis components is 5.8 min for cefaclor, 7.0 min for cephalexin and 10.4 min for ampicillin correspondingly. The peak with Rt 2.44 min corresponds to the by-product of the reaction—DPG.

(Fig. 1). The level of transformation of corresponding key amino acids was 50–70%. The composition of amoxicillin, cephadroxil and so named procefoperazone—semi-product in cefoperazone synthesis—was synthesized analogically with using of the same biocatalyst, corresponding key amino acids—6-APA, 7-ADCA, and TET-7-ACA, and the methyl ester of DHPG as acylating agent.

So, the availability of the enzymes and comprehension of the mechanism of their action are the base of a new approach to screening of modern drugs—"combinatorial enzymology". The methods of combinatorial enzymology allow to design and produce the hits and leads, using as a building blocks different key amino acids and acylating agents, which are the substrates for used enzymes.

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