Chemistry of Natural Compounds, Vol. 37, No. 2, 2001

ANTIBIOTICS PRODUCED BY *Bacillus* BACTERIA

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UDC 615.33:576.851.5

Data for antibiotics produced by Bacillus *bacteria and structural formulas of the antibiotics gramicidin, tyrocidine, bacitracin, mycobacillin, surfactin, bacilysin, and subtilin are presented. Various biosynthetic mechanisms and chemical properties are discussed. The lantibiotics, which are produced by certain* Bacillus *species and have unique structural features different from those of other antibiotics, are analyzed. Structural formulas of unusual amino acid residues in lantibiotics are presented and their mechanisms of biological action are examined.*

Key words: *Bacillus,* antibiotics.

Aerobic *Bacillus* bacteria are widely distributed in nature [1-4]. They participate in various biological processes and are very resistant to various physicochemical actions of microorganisms [5, 6]. They can also adapt to environmental changes [7-9].

Representatives of the *Bacillus* genus and biologically active metabolites produced by them in addition to their practical application in various branches of the economy have been widely studied in the scientific literature [10-21]. This confirms that continued comprehensive research on them is advisable.

The majority of studied antibiotics produced by *Bacillus* strains are polypeptides of low molecular weight [14, 22-24] that are synthesized by ribosomal or nonribosomal mechanisms.

In 1946, a strain producing gramicidin C was isolated from soil near Moscow and described as *B. brevis* var. G.B. [25]. The producer of gramicidin C, *B. brevis* var. G.B., spontaneously dissociated into several forms during development in a liquid nutrient medium. These differed in the morphology of the colonies and other properties during growth on yeast-extract solid media. The resulting varieties represented the following forms: reticulated (R), smooth (S), and two planar forms (P^+ and P^-) [10]. The new varieties of *B. brevis* had different characteristics than the starting culture, including the ability to produce antibiotics. Only two forms could produce gramicidin C, R and P^+. Forms S and P^- do not produce this antibiotic. Similar results were obtained by us during a study of the endophytic strain *B. subtilis* N, which also dissociated into R- and S-forms. However, both dissociated forms exhibited distinct antimicrobial activity although they differed substantially in antagonistic activity [26].

Four polypeptide antibiotics were isolated from the gramicidin fraction of tyrothricin: gramicidins A, B, C, and D. The amount of gramicidin A was found to be predominant during separation of the gramicidin fraction [10]. Some preparations of the gramicidin fraction contained 85% gramicidin A, 9% B, 6% C, and traces of D. A difference between gramicidin C and tyrothricin was observed during isolation of the antibiotic. Gramicidin C was easily converted into needle-like crystals during evaporation of the alcohol extract obtained from the bacterial mass whereas tyrothricin under analogous conditions was amorphous. It has been concluded that the biosynthesis of gramicidin C by the varieties that form antibiotics (R and P^+) occurs independently of spore formation. The antibiotic is formed both during spore formation and by actively multiplying cells. However, biosynthesis of gramicidin does not occur during transition of the culture to mass spore formation [10]. The results of our research have showed that biosynthesis of antibiotics stops at the start of sporulation of strains 23 and N of *B. subtilis* [6, 9].

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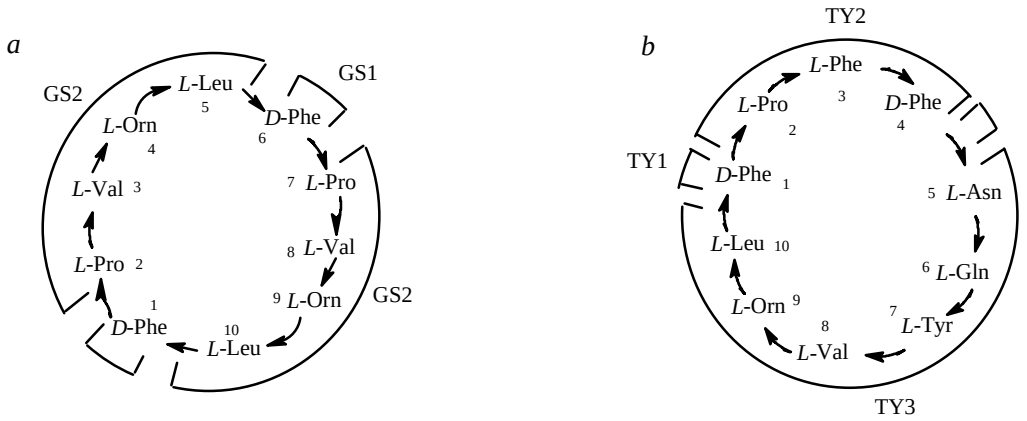

Fig. 1. Structural formulas of gramicidin C (a) and tyrocidine (b). Amino acids activated by multifunctional enzymes gramicidin C synthetase 1 (GS1) and 2 (GS2) and tyrocidine synthetase 1 (TY1), 2 (TY2), and 3 (TY3) are included in brackets.

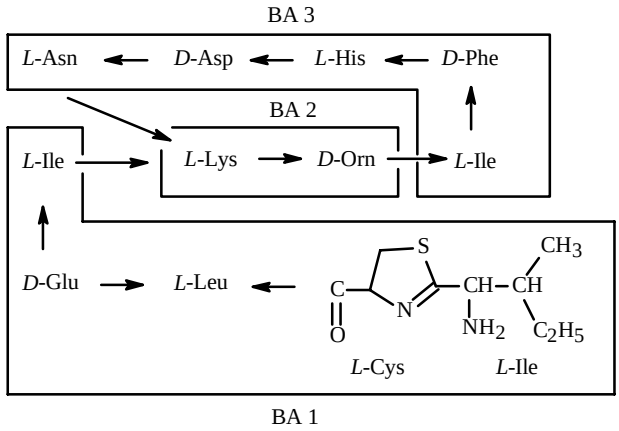

Fig. 2. Structural formula of bacitracin A. Amino acids activated by bacitracin synthetases BA1, BA2, and BA3 are surrounded by lines.

Gramicidin C (M-1269) and tyrocidine (M-1300), which are produced by *B. brevis* ATCC999 and 8185, respectively [24], are synthesized by a nonribosomal but multi-enzyme thiotemplate mechanism and are small cyclic peptides (Fig. 1). Gramicidin C consists of two identical pentapeptides joined head-to-tail. The synthesis of gramicidin C is catalyzed by the multifunctional enzyme complex "gramicidin C synthetase," which consists of two enzymes, gramicidin synthetase 1 (GS1) and gramicidin synthetase 2 (GS2) [27].

The mechanism of tyrocidine biosynthesis is similar to that of gramicidin C. Tyrocidine is a cyclic decapeptide with an amino acid sequence identical to that of gramicidin C (L-Val-L-Orn-L-Leu-D-Phe-L-Pro). However, its sequence is interrupted by five other amino acids (Fig. 1). The biosynthesis of tyrocidine consists of three enzyme fractions: tyrocidine synthetase 1 (TY1, 100 kDa), tyrocidine synthetase 2 (TY2, 230 kDa), and tyrocidine synthetase 3 (TY3, 440 kDa).

The peptide antibiotic bacitracin (M-1410), which is produced by certain *B. licheniformis* strains [28], is also synthesized by a multifunctional thiotemplate mechanism. Three multifunctional enzymes, AB1 (335 kDa), BA2 (240 kDa), and BA3 (380 kDa) catalyze bacitracin A synthesis (Fig. 2).

The biosynthesis of the cyclic antifungal antiobiotic mycobacillin (M-1590), which is produced by strain B3 of *B. subtilis*, is an alternative to the thiotemplate mechanism of peptide antibiotic biosynthesis [24, 29]. Instead of two-step activation of amino acids, a single-step mechanism with formation of aminoacylphosphate was proposed. The enzyme complex that catalyzes the synthesis of mycobacillin is separated into three fractions: A, which operates during synthesis of the first pentapeptide; B, which catalyzes the synthesis of the nonapeptide; and C, which catalyzes synthesis of the final product (Fig. 3).

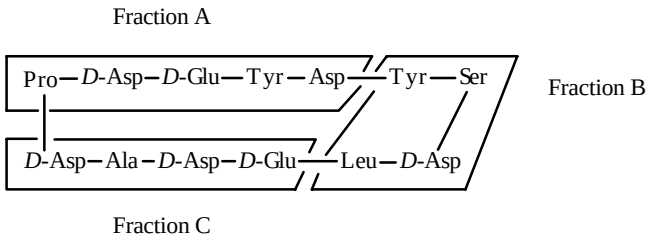

Fig. 3. Structural formula of mycobacillin. Amino acids activated by three fractions of mycobacillin synthetase are surrounded by lines.

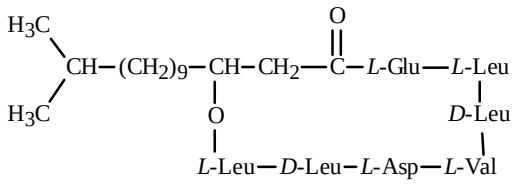

Fig. 4

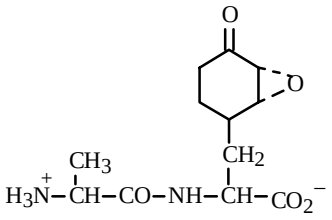

Fig. 5

Fig. 4. Structural formula of surfactin.
Fig. 5. Structural formula of bacilysin.

The lipopeptide antibiotic surfactin (M-1036) is of nonribosomal origin. However, it may be synthesized by a mechanism that differs from the multi-enzyme thiotemplate one of gramicidin C, tyrocidine, and mycobacillin (Fig. 4). Amino-acid precursors are activated by an ATP-Pi exchange reaction during biosynthesis of surfactin. However, all amino acids are activated, in contrast with the biosynthesis of mycobacillin [30]. Surfactin was first isolated from *B. subtilis* ATCC 1332 culture [31]. It is known as a strong biosurfactant (decreases the surface tension from 72 to 27 mN/m) and inhibitor of Ca- and Mg-dependent cyclic adenosine 3′,5′-monophosphate phosphodiesterase, which was isolated from the filtrate of *B. subtilis* culture [32]. An interesting hypothesis was made on the basis of the research results. The inhibition is caused by chelation of free carboxylic groups of glutamic and aspartic acids in the peptide. The results of our comparative observations of soil and endophytic strains *B. subtilis* 23 and N, which were isolated from various ecological abnormalities, suggested that they also produce identical surfactin-like antibiotics [19]. We purified the biological substances using a generally accepted scheme: centrifugation of the culture liquid and extraction from the supernatant liquid by $CHCl_3$, which was then evaporated in a rotary evaporator. The antibiotic substances were eluted from the resulting solid with 80% ethanol. Then, the extract was fractionated by TLC with elution by 80% ethanol of the ninhydrin-positive fraction with R_f 0.72 that has antimicrobial activity. It should be noted that the alcohol extracts that were obtained in this manner were stored at 4°C and did not lose biological activity for one year. Separation of the antibiotics produced biologically active substances (850 mg per liter of 3-day culture liquid of strains 23 and N).

Bacilysin (M-270) is one of the simplest peptide antibiotics (Fig. 5). It is produced by strain *B. subtilis* Marburg 168 and is a dipeptide containing L-alanine and the unusual amino acid L-anticapsine [33]. It was demonstrated that bacilysin is synthesized from amino acid precursors in the presence of ATP and Mg^{2+} [34].

Subtilin (M-3317) is a well-known antibiotic of ribosomal origin. It belongs to group A of lantionine-containing antibiotics, lantibiotics, the biochemical features and mechanism of biological action of which will be described below. The subtilin precursor is synthesized in ribosomes and undergoes extensive post-translation modification, which includes dehydration of threonine and serine units into dehydroforms. These are joined to cystine units in the subtilin polypeptide chain (Fig. 6). As a result, the 32-amino-acid antibiotic subtilin is formed [35].

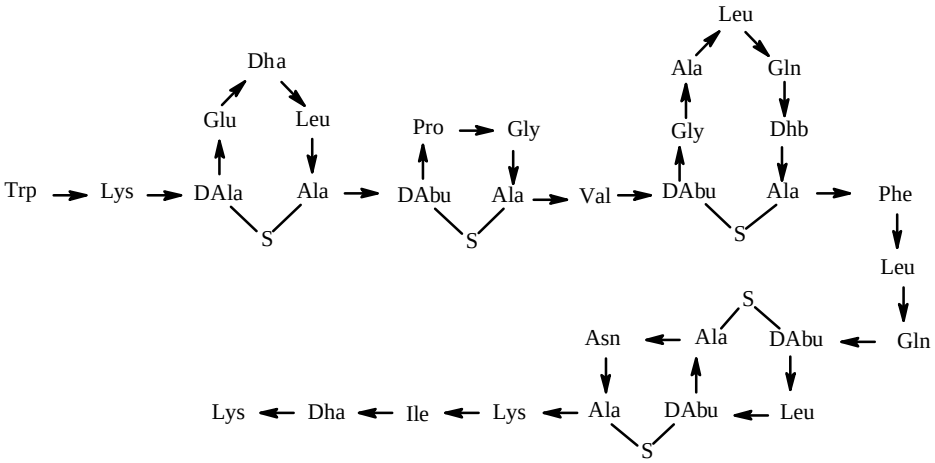

Fig. 6. Structural formula of subtilin. Dha = dehydroalanine; Dhb = dehydrobutyrin (β-methyldehydroalanine), DAbu = aminobutyric acid, Ala-S-Ala = linthionine; DAbu-S-Ala = β-methyllanthionine.

Autoclaved subtilin retains completely its antibacterial activity. Furthermore, subtilin has been found to be highly stable at high temperature and low pH values [24].

Molecular-genetic and biochemical analyses and DNA sequencing showed that the biosynthesis genes of several antibiotics are located in the polycistronic transcriptional units and are controlled by a single regulatory system that directs gene expression when *Bacillus* cells reach a steady-state growth stage [9, 35, 36]. A study of the biological role of low-molecular-weight antibiotic peptides during sporulation found that in most instances the antibiotics were produced at the same point in the growth cycle and under conditions where sporulation began [24, 37]. A study of mutant strains incapable of sporulation found that the mutant bacteria did not exhibit antibiotic activity [38]. This can be explained by the existence of parallel regulatory pathways. However, this hypothesis was not confirmed because other scientists [39] observed that mutant strains incapable of producing tyrocidine or linear gramicidin were asporogenic and could sporulate on nitrite-free medium with tyrocidine added to it but did not sporulate on enriched medium. Gramicidin S-negative mutants *B. brevis* ATCC9999 are thermally sensitive and have a low level of dipicolinic acid. However, they grew for 80 min whereas this process extended for 8-10 h for normal cells [40]. However, examples are also known that contradict the hypothesis that antibiotics play an important role in cellular differentiation of bacilli. For example, certain gramicidin C-negative strains sporulated normally [41].

The term "lantibiotics" has appeared in the scientific literature. It refers to antibacterial peptides that differ from other antibiotics by having unique structural features [35, 42]. It should be noted that this term was proposed instead of an abbreviation for lantionine-containing peptides in order to indicate their own features, i.e., the presence of the sulfoamino acid lantionine and antibacterial activity [14]. In addition to lantionine and its analog 3-methyllantionine, all lantibiotics contain the unsaturated amino acids didehydroalanine and didehydrobutyrine (Fig. 7). Some lantibiotics may also contain unusual amino acids such as S-aminovinylcysteine, erythro-3-hydroxyaspartic acid, and lysinoalanine [42]. Lantibiotics are divided by general structure, molecular weight, and charge into groups A and B (Table 1). Lantibiotics of group A have positive charges from 2+ to 7+ whereas lantibiotics of group B have comparatively small charges. Lantibiotics of group A are produced by lactococci, lactobacilli, staphylococci, streptococci, and *Bacillus* bacteria. They are described as elongated linear peptides with molecular weight 2100-3500 Da. This group includes nisin, a lantibiotic that is widely used as a preservative; subtilin, the biosynthesis of which we discussed earlier; epidermin; gallidermin; mersacidin; and astagardin [14, 43].

A study of the conformation of group A lantibiotics determined the limits of their conformational freedom. A study of nisin and Pep5 in aqueous medium found that lantibiotics of group A are rather flexible molecules.

Lantibiotics of group B are produced only by streptococci. They are globular with molecular weight 1800-2100 Da. They typically have weak antibacterial activity [43, 44]. However, they are of scientific interest. For example, cinnamicin and duramicins are strong inhibitors of phospholipase A_2 and promising substances for curing allergic diseases. They can also be used to create medicinal preparations such as blood-pressure regulators. Conformational studies of lantibiotics of group B found that they are highly rigid molecules and form almost globular and densely packed molecules. Application of NMR spectra suggested the existence of specific interactions between phospholipids and hydrophobic and hydrophilic parts of a lantibiotic [45].

TABLE 1. Classification of Lantibiotics in Groups "A" and "B" According to Charges, Conformation, and Biological Activity [14]

Lantibiotic	Mol. wt., Da	Number of amino acids	Unusual amino acids					Charges			Properties
			Lan	MeLan	Dha	Dhd	others	positive	negative	total	
Group "A" lantibiotics											
Pep 5	3488	34	2	1	-	3	-	8	1	7+	Elongated, helical,
Nisin	3353	34	1	4	2	1	-	4	1	3+	cationic, amphiphilic,
Subtilin	3317	32	1	4	2	1	-	4	2	2+	energy-dependent
Epidermin	2164	32	2	1	-	1	Cys(Avi)	3	0	3+	membrane
Gallidermin	2164	22	2	1	-	1	Cys(Avi)	3	0	3+	pore formers
Mersacidin	1825	20	-	4	1	-	Cys(Avi)	3	1	0	Amphiphilic,
Astagardin	1890	19	1	3	-	-	-	1	2	1-	hydrophobic
Group "B" lantibiotics											
Cinnamycin	2041	19	1	2	-	-	HyAsp, LysN-Ala	3	2	1+	Compact, almost
Duramycin	2012	19	1	2	-	-	HyAsp, LysN-Ala	3	2	1+	neutral, amphiphilic
Duramycin B	2012	19	1	2	-	-	HyAsp, LysN-Ala	3	2	1+	enzyme inhibitors,
Duramycin C	2012	19	1	2	-	-	HyAsp, LysN-Ala	3	2	0	immunologically active
Ancavenin	1959	19	1	2	1	-	-	2	2	0	

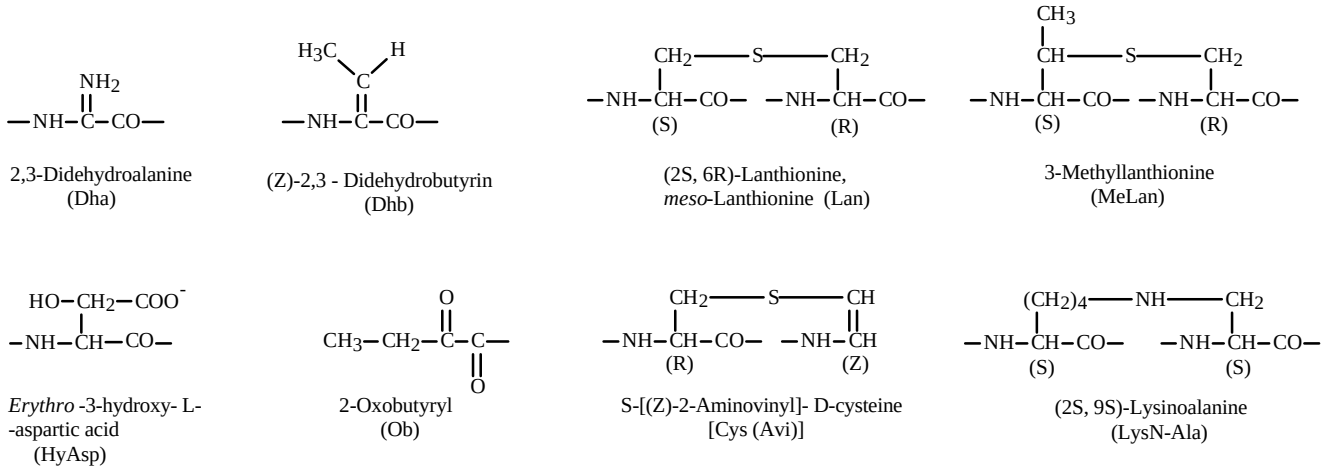

Fig. 7. Structural formulas of unusual amino acids observed in lantibiotics.

A study of the biosynthesis of lantibiotics indicates that they are coded by certain genes and translated as prepeptides. Then they undergo post-translational modifications [46]. The prepeptides of A lantibiotics are similar to each other in having an unusual N-terminal leader peptide and a C-terminal propeptide. The leader peptides are highly hydrophilic with an overall negative charge and a preliminary *p*-spiral conformation. The leader and prepeptide regions are bonded to each other by a twisted region that contains a characteristic region of proteolytic separation. The prepeptide is modified by a series of reactions including dehydration of hydroxyamino acids, formation of sulfo groups, and cleavage of the leader peptide. Individual lantiboitics may undergo subsequent modifications. Furthermore, enzymes that catalyze these transformations are organized into an operon [14].

The biological activity of group A lantibiotics is based on the formation of energy-dependent short-lived pores [14]. Rapid release of tracers over one minute was observed upon treatment of cells of gram-positive microorganisms containing labeled amino acids with micromolar concentrations of A lantibiotics [23, 47]. ATP was also observed outside the cells, which indicates that pores of a certain size were formed and membranes were not disrupted. An estimation of the pore diameters based on the hypothesis that cylindrical pores were formed, the length of which is equal to the membrane thickness, gave pore dimensions of about 1 nm for nisin [14] and Pep5 [48] and 2 nm for subtilin [23]. Pore of this diameter can pass ions of amino acids and ATP. Channels are formed from several molecules, the number of which can vary. Peptides are bound through ionic interactions of positive charges of the lantibiotic and negative charges of the phospholipid head groups [49]. This stabilizes the elongated spiral structure of the polypeptide that is observed in NMR experiments.

According to several investigations, cells of gram-positive bacteria are insensitive to the antimicrobial action of lantibiotics. This may be due to the presence of an outer membrane that does not pass peptides of this size. On the other hand, nisin and Pep5 in millimolar concentrations had little effect on eukaryotic cells, probably because of physical destabilization of the membrane [14]. The cells and membranes became sensitive through osmotic shock upon penetration of lantibiotics through the outer membrane [50].

Gaps and contradictory data may be encountered in the literature and generate several new issues. Therefore, the study of the biology of antibiotic synthesis by *Bacillus* representatives is actually only beginning and has great potential for basic scientific studies.

Therefore, further research and investigations of new producers of novel antibiotics among *Bacillus* representatives and the biosynthesis of known antibiotics, the determination of their chemical properties, and the mechanism of their biological action are of fundamental and practical interest.

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