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A bibliographic review on aminonucleoside antibiotic A201A
Una cerca bibliogràfica sobre l'antibiòtic aminonucleosídic A201A

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January 2020

Universitat de Barcelona

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Hi ha una força motriu més poderosa que el vapor, l'electricitat i l'energia atòmica: la voluntat.

Albert Einstein

Vull agrair a en Jordi Robles per haver-me transmès gran quantitat de coneixements durant aquest període, per tot l'ajut i consells que he rebut. També per haver dedicat el seu temps i esforç en resoldre tots els meus dubtes amb molta rapidesa i ajudar-me a entendre els continguts.

Als meus pares, la meva germana i la meva parella per haver-me donat sempre suport en aquells moments de dubte i la seva eterna confiança.

Als meus companys de carrera per fer tot el procés més entretingut i a totes aquelles persones que m'han recolzat en diferents aspectes, gràcies.

REPORT

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1. SUMMARY

Currently, one of the most important challenges of medicinal chemistry is the fight against drug-resistant infections. The increase in bacterial resistance to existing antibiotics, together with the decrease in research and development spending in the pharmaceutical industry, is becoming a major threat to global health. The reasonable use of antibiotics could slow down the development of bacterial resistance, but it is essential to continue research to find new and more efficient drugs. Within this context, natural products continue to be a source of inspiration for new targets and drugs.

A201A is a natural antibiotic recently discovered that derives from adenosine, and comprises two units of glycoside and one of coumarate. Notably, A201A results from the combination of two known antibiotics with potent antibacterial activity, such as hygromycin A and puromycin. Similarly to these two drugs, A201A also acts by inhibiting protein synthesis by blocking translation in the ribosome.

The main objective of this present TFG report was a review on A201A, with a focus on the synthetic routes of the compound and its derivatives, the elucidation of the biosynthesis pathway, and studies of structure-antibacterial activity.

Keywords: aminonucleoside, antibiotics, biosynthesis, organic synthesis, protein synthesis inhibition.

2. RESUM

Actualment, un dels reptes més important de la química medicinal és la lluita contra les infeccions resistents als medicaments. L'augment de la resistència bacteriana als antibiòtics existents, juntament amb la disminució de la despesa en investigació i desenvolupament a la indústria farmacèutica, s'està convertint en una amenaça important per a la salut mundial. L'ús raonable d'antibiòtics podria frenar el desenvolupament de resistència bacteriana, però és essencial continuar amb la investigació per trobar fàrmacs nous i més eficients. En aquest context, els productes naturals continuen sent una font d'inspiració per a nous objectius i fàrmacs.

L'A201A és un antibiòtic natural descobert recentment que deriva de l'adenosina i inclou dues unitats de glicòsid i una de cumarat. En particular, A201A resulta de la combinació de dos antibiòtics coneguts, amb una potent activitat antibacteriana, com són la higromicina A i la puromicina. De manera similar a aquests dos fàrmacs, l'A201A també actua inhibint la síntesi de proteïnes bloquejant la traducció al ribosoma.

L'objectiu principal d'aquest present informe de TFG ha estat una revisió sobre A201A, amb una atenció centrada en les rutes sintètiques del compost i els seus derivats, l'elucidació de la via de biosíntesi i estudis d'activitat antibacteriana-estructura.

Paraules clau: A201A, aminonucleòsid, antibiòtics, biosíntesi, inhibició de la síntesis proteica, síntesis orgànica.

3. INTRODUCTION

Antibiotics are natural or artificial chemical synthetic products, designed to selectively block some crucial process in microbial cells. Most of the classes of antibiotics in use were discovered more than forty years ago and since then a large majority of the newly approved drugs have been based on chemical modifications of existing structures to combat clinical resistance. To date, all kinds of antibiotics have seen the emergence of resistance in bacteria that compromise their use.[1] At present, antibiotic resistance is today one of the greatest threats to global health, food security and development. It is a natural and inevitable phenomenon, since it occurs when bacteria mutate in response to the use of antibiotics, but the misuse of these drugs in humans and animals is accelerating the process. In the last two decades, the resistance of pathogens to common antibiotics has increased 100%. In addition, bacterial conjugation has caused multiresistance to different antibiotics. Consequently, treatment of certain infectious diseases has become more difficult due to the loss of efficacy of antibiotics.[2] Antibiotic resistance prolongs hospital stays, increases medical costs and mortality among patients.

Meanwhile, it is necessary to change urgently the way of prescribing and using antibiotics in order to sustain the actual antibiotic battery. Although, new medications are being developed, if current behaviors are not modified, antibiotic resistance will continue to pose a serious threat. Behavioral changes are also needed and should include measures to reduce the spread of infections. Because the achievements of modern medicine are being put at risk, if we do not have effective antibiotics to prevent and treat infections, organ transplants, chemotherapy, and surgical interventions will become more dangerous. Therefore, it is urgent and necessary to implement new policies and invest in research to develop new strategies to combat bacteria with novel antibiotic compounds.[3]

Motivated for this urgency, researchers and scientists are trying to create new antibiotics, but this process takes much more time than bacteria need to develop new resistance mechanisms. It has been estimated that developing an antibiotic involves about 15 years and a large investment. Since antibiotics drugs are commonly used in short-term treatments,[4] the big pharmaceutical companies have neglected the antibiotic research due to low profits and

directed their investments to other disease areas.[5] Other big problem is that there are countries that can not allocate enough money to fight antibiotic resistance. Studies conducted by economists looking to the future indicate that if the situation continues the same course it is expected that in 2050 there will be ten million deaths annually in the world due to resistance bacteria, a very considerable increase in comparison with 700,000 deaths annually in 2016.[6] The sequencing of the first complete bacterial genome in 1995 generated new hope for the discovery of antibacterial drugs.

Most of the antibiotics introduced in human clinical use have been natural products made by a microorganism to affect neighboring microbes, either to regulate their growth or to activate their elimination. When antibiotic producers enter the stationary phase and face competition from nutrients and/or space, they activate the genes that encode antibiotic molecules to wage a chemical war against their neighbors. Actinomycetes have been, for decades, one of the most important sources for the discovery of new antibiotics with a significant amount of drugs and analogs successfully introduced in the market and still used today in clinical practice.

Among the compounds produced by the actinomycete group bacteria, A201A noted for being a complex aminoacyl nucleoside antibiotic (Fig. 1), which was first isolated from *Streptomyces Capreolus* NRRL 3817 in 1985.[7] Interestingly, it has also been reported that the same nucleoside antibiotic was isolated from crude extracts of a deep-sea bacteria in the ocean of southern China, *Marinactinospora thermotolerans* SCSIO 00652 in 2011.[8] The structure of A201A was elucidated by NMR[7] (see appendix) and, recently, the compound was fully synthesized chemically by Yu's group.[9]

A201A is very active against gram-positive aerobic and anaerobic bacteria, as well as most gram-negative anaerobic species. However, it is much less toxic to aerobic gram-negative bacteria, some fungi and mammals.

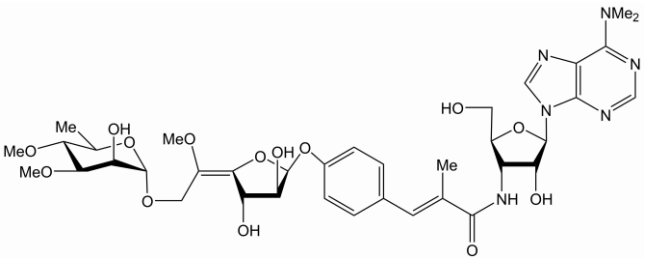


Figure 1. Chemical structure of A201A

3.1. HOW THE ANTIBIOTIC A201A WORKS

The genetic information is translated into proteins in the ribosome. Research on antibiotics that act on ribosome has provided much needed information about its mechanism of action.[10] Current structural studies have characterized antibiotics that had been discovered decades ago, with unique structural characteristics that use novel modes of action to interact with the ribosome and thus inhibit the translation of genetic information. Consequently, ribosome inhibitors are successful medications to treat infections.[11]

The ribosome translates the genetic information encoded in mRNA into proteins. Bacterial 70S ribosome consists of three stands of RNA and more than 50 proteins assembled into two subunits: a small 30S and a large 50S. The peptidyl transferase center (PTC) where peptide building occurs consists of three sites (Fig. 2A)[12] : Aminoacyl-, Peptidyl- and Exit-site, which are the positions that the entering Aa-tRNAs and the proteins that are being constructed occupy into the ribosome. The tRNA bound to the amino acid to be incorporated is first positioned in the A-site, then the translocation occurs to the P-site where the protein chain is elongated. Finally, when the peptide is completed it is transferred to the E-site to be expelled from the ribosome.

Antibiotics that target the large subunit, such as the A201A antibiotic, tend to cluster at the PTC, the peptide bond formation site, which occurs between Aa-tRNA at A-site and P-tRNA at P-site with the result of inhibiting the proper formation of peptide bonds by steric blocking.[13]

The chemical structure of A201A has similarities with hygromycin A and puromycin antibiotics, so that A201A showed to bind analogously to them within the PTC of the large ribosomal subunit (Fig. 2B). A201A has an additional D-rhamnose moiety compared to hygromycin A that allowed to reach the ribosome more deeply. Despite this, X-ray crystal studies showed that A201A proceeded by a different mechanism than hygromycin A and puromycin. A201A occluded the A-site by causing local distortions to the tRNA acceptor arm that avoided the proper housing of Aa-tRNA at PTC, with the result of inhibiting the formation of peptide bonds.[14, 15]

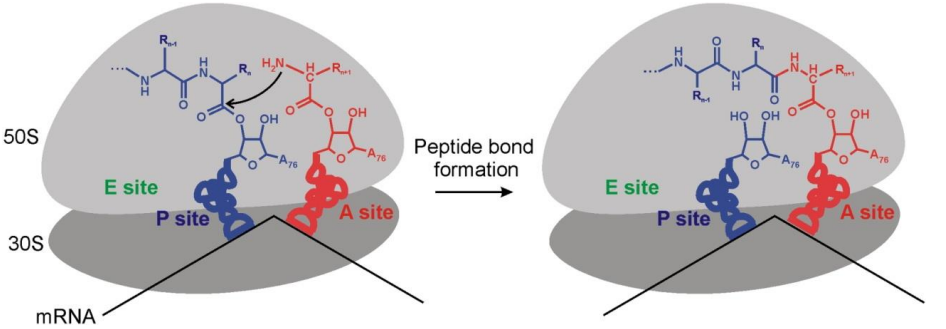


Figure 2A. The translocation process during the ribosomal protein synthesis. The image was reproduced unaltered from reference no. 12.

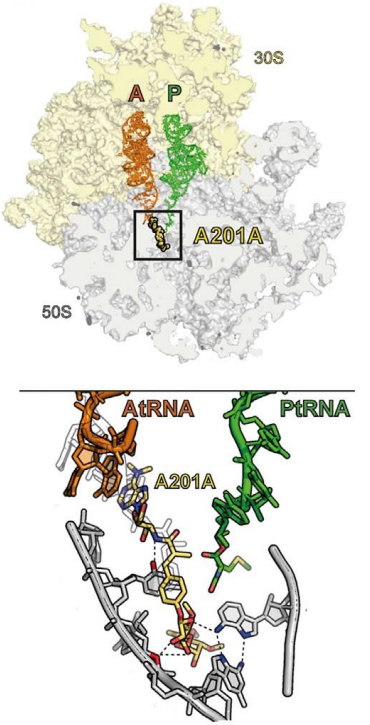


Figure 2B. X-ray structure of the binding of A201A within the bacterial ribosome, and enlarged image of the interaction. The image was reproduced unaltered from reference no. 13.

4. OBJECTIVES

As mentioned above, antibiotic resistance is becoming one of the largest threats to human health which urges to increase the efficiency of existing drugs, and seek also new strategies and antibiotics to fight bacteria. It is also very important to raise public awareness of antibiotic abuse for human use and animal feeding.

In this context, the main objective of this work was to gather information about a new antibiotic named A201A with a structure similar to two existing antibiotics, puromycin and hygromycin A, and which showed promising wide-spectrum antibacterial activity.

In particular, this report covered three main issues:

- Review of the organic synthetic routes leading A201A.
- Summary of the biosynthetic pathways that were proposed to explain the formation of A201A in the producing bacteria.
- Structure-antibacterial activity relationships of A201A and its analogs.

5. METHODS

In this bibliographic work on A201A antibiotic, different databases were used which were accessible through the services of CRAI-UB.

First of all, an extensive search was carried out by using A201A as a searching keyword. Starting with the American Chemical Society (ACS) database, 5 articles were found. It was followed by searching Web of Science database, including the citations of the primary collected articles, which produced 18 additional references. Reaxys database was also used in the same way as the other two databases, also searching for chemical structure similarity with A201A (higher than 60%), and about 40 articles were collected. The same searching process was repeated via Scifinder database, which also incorporated the search of the chemical reactions in which A201A participated, resulting in more than 50 references. We also searched in Index Merck and Pharmacopeia databases but no more additional references were found.

Finally, the number of references were narrowed down by using more specific keywords such as Actinomycetes, antibiotics, bacterial resistance, biosynthesis, pharmaceutical drug, etc., in order to focus on the objectives that were established at the beginning of the work.

6. A201A: SYNTHESIS, BIOSYNTHESIS AND ANTIBACTERIAL ACTIVITY

6.1. SYNTHESIS

Although, several synthetic procedures were known for preparing chemically close compounds, such as puromycin and hygromycin A,[16-18] at the present time the synthesis of A201A was reported only once by the group of Shenyou and col.[9]

The synthesis devised by Shenyou et al.[9] to synthesize the antibiotic A201A (Figure 3) comprised the five building blocks depicted in Figure 3, namely, 3,4-di-*O*-methyl-D-rhamnose **1**, an uncommon hexafuranose **2**, α-methyl-*p*-coumaric acid **3**, 3-amino-3-deoxyribose **4**, and 6-dimethylaminopurine **5**. These units are connected with different linkages, two *O*-glycosidic, one β-glycosidic and one amide. The overall synthesis was divided into three main parts.

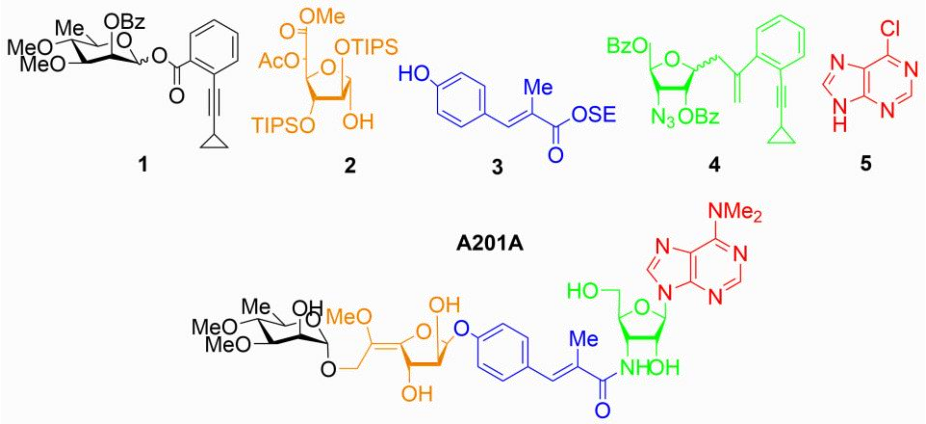
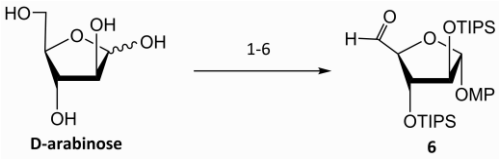


Figure 3. Synthetic fragments for preparing **A201A** according to the route devised by Shenyou et al.[9]

6.1.1. First part: Furanose construction

The route started by synthesizing the furanose **6** from D-arabinose, as it contains the configuration at C-2 and C-3 that was present in A201A. D-arabinose was transformed into compound **6** after five protection steps and a final selective oxidation of the primary alcohol into aldehyde (See Figure 4).



1.- MPOH, TMSOTf, 80%. 2.- NaOMe in MeOH/DCM at rt, 99%.

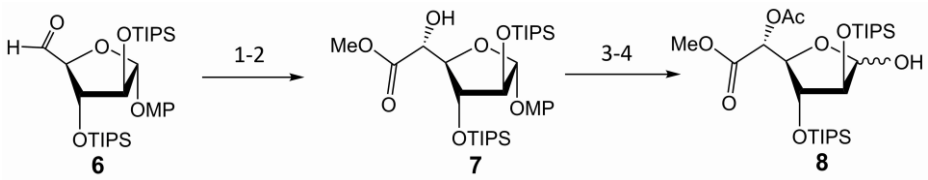
3.- MTrCl, Pyr, 77%. 4.- TIPSOTf, 2,6-lutidine in DCM at rt, 91%.

5.- $BF_3{\cdot}OEt_2$, Et_3SiH -78ºC to 20ºC, 78%. 6.- IBX in DMSO at 80ºC, 93%.

Figure 4. Transformation of D-arabinose into protected furanose **6**.

Furanose **6** was next treated with ^{t}BuLi in the presence of $(PhS)_3CH$ followed by $CuO/CuCl_2$ to produce ester **7** (Figure 5). In the first reaction, ^{t}BuLi reacted with $(PhS)_3CH$ to produce $(PhS)_3CLi$ which made a nucleophile attack over aldehyde to produce a phenylthio-ortoester adduct, that was finally transformed into monoester **7** by $CuO/CuCl_2$ treatment.[19]

Then, the nascent OH on **7** was protected with an acetyl group. It was followed by the selective removal of the *p*-methoxyphenyl group (MP) with ceric ammonium nitrate in aqueous MeCN to produce the hemiacetal **8** (Figure 5).



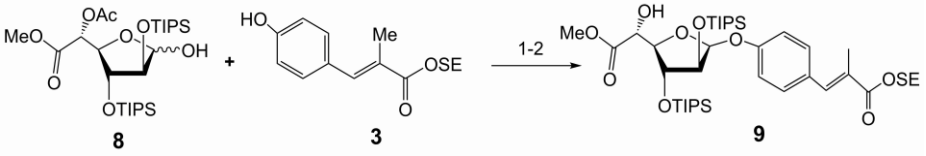
1.- $(PhS)_3CH$, ^{t}BuLi in THF at -78ºC, quantitative. 2.- $CuO/CuCl_2$ in $MeOH/CH_2Cl_2/H_2O$, 78%.

3.- Ac_2O, Et_3N, CH_2Cl_2 at rt, 96%. 4.- CAN in $MeCN/H_2O$ at 0ºC to rt, quantitative.

Figure 5. Transformation of aldehyde **6** into **8**, by ester formation and alcohol protection by acetylation.

6.1.2. Second part: Formation of the glycosidic bond between furanose *8* and α-methyl-*p*-coumaric acid *3*.

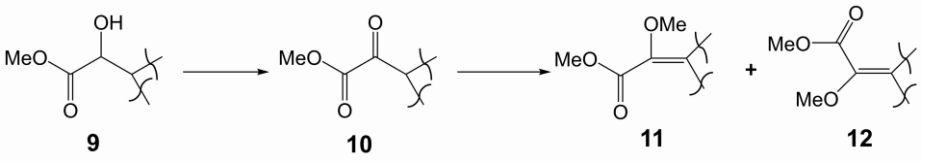
Furanose **8** and α-methyl-*p*-coumaric acid **3** were first condensed by a Mitsunobu reaction, to form an *O*-glycosidic bond and it was followed by a treatment with K_2CO_3 in aqueous MeOH to remove the acetyl group leading to acetal **9** (Figure 6).[20]



1.- PPH_3, DIAD in toluene at 60ºC, 79%. 2.- K_2CO_3 in $MeOH/H_2O$, 82%.

Figure 6. Formation of acetal **9** by *O*-glycosidic bond formation and deprotection of alcohol.

Afterward, the hydroxyl group was oxidized to ketone **10** with the hypervalent iodine compound IBX. Then, a mixture of the isomeric methyl enol ethers **11-12** were obtained by reacting with Cs_2CO_3 and Me_2CO_3 in MeCN (Figure 7). Finally, the TIPS and SE protecting groups were eliminated with TASF in DMF, thus obtaining the compound **13** (Figure 8).



1.- IBX in DMSO at 80ºC, 95%. 2.- Cs_2CO_3, Me_2CO_3 in MeCN at rt, 69%.

Figure 7. Conversion of hydroxyl ester **9** into enol methyl ethers **11-12**.

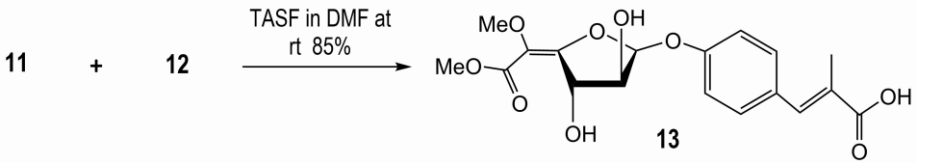


Figure 8. Transformation of enol methyl ethers **11-12** into compound **13** by deprotection.

6.1.3. Third part: Completion of the synthesis of A201A

At this stage, two glycosyl ortho-alkynylbenzoates, compounds **4** and **1**, were needed to form the two glycosidic bonds present in A201A. These intermediates were prepared from D-xylose and D-mannose, respectively, following previously reported procedures.[21] The

cyclopropylethynylbenzoate group present in **4** and **1** was adopted after extensive work to optimize the Au(I) catalyzed procedure that was chosen by authors for glycosidations.

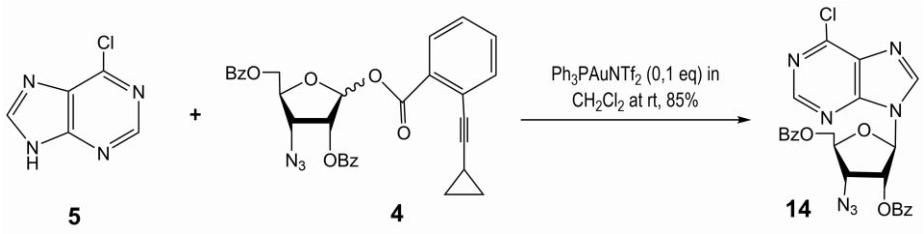


Figure 9. Formation of the glycosidic bond between **4** and **5** catalyzed by gold(I).

In the first glycosidation step, nucleoside **14** was produced by β-glycosidic bond formation between the ribose **4** and purine **5** by $Ph_3PAuNTf_2$ catalysis (Figure 9).[22] The mechanism that was proposed for Au(I) catalyzed glycosidation is depicted below in Figure 10.[23] The interaction of Au(I) catalyst with the triple bond of the ortho-alkynylbenzoate (Figure 10, **A**) was crucial for the success of the glycosidation, providing the oxonium cation in mild conditions, (Figure 10, **B**). This oxonium cation condensed with chloropurine **5** to produce nucleoside **14**, while at the same time the Au(I) catalyst was regenerated by protodemetalation, (Figure 10, **C**). This Au(I) catalyzed step had significative benefits according to the authors: the reaction resulted in generally excellent yields, it was conducted in neutral and mild conditions, the side reactions were minimal and the ortho-alkynylbenzoates were stable substrates.

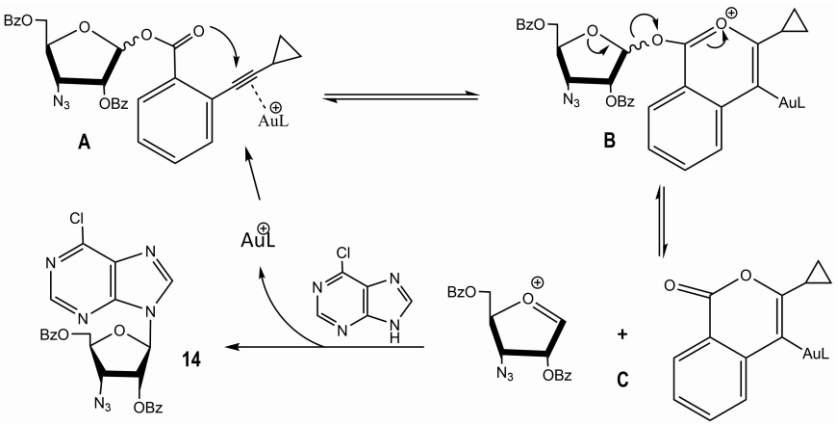
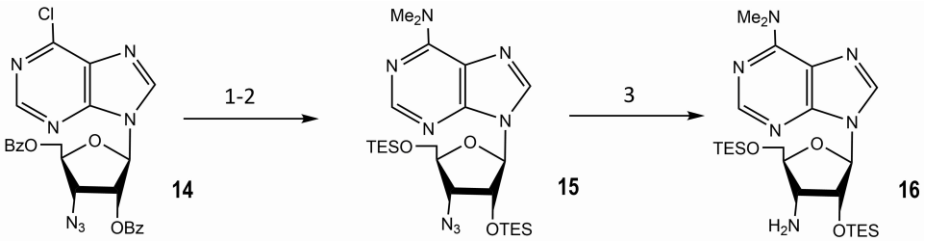


Figure 10. Gold(I)-catalyzed mechanism of glycosidation.

Nucleoside **14** was further processed to obtain intermediate **16**. First, 6-chloride was substituted by dimethylamine. Next, the benzoyl groups were removed and the hydroxyl groups were subsequently reprotected with the TES group, to yield **15**. 3'-Azide at **15** was further transformed into primary amine 3'-amino-3'-deoxyadenosine **16** by reduction with PPh_3 in THF/H_2O (Figure 11).

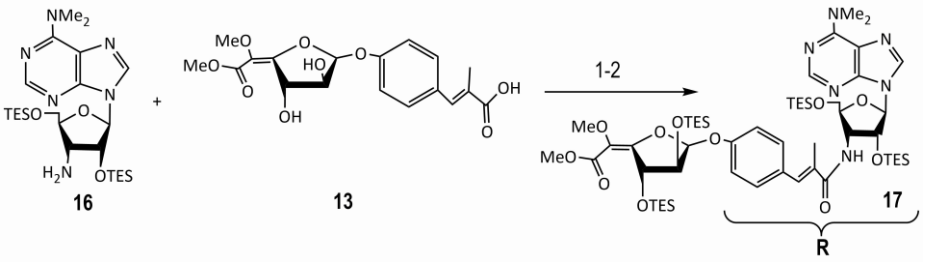


1.- aq. Me_2NH in EtOH, 90ºC, quantitative. 2.- TESOTf in pyridine at rt, 76%.

3.- PPh_3 in THF/H_2O, SiO_2 at 50ºC, 93%.

Figure 11. Transformation of nucleoside **14** into **16**, by chloride replacement with dimethylamine, reprotection of hydroxyls with TES group and reduction of azide into amine.

Nucleoside **16** was then condensed with the compound **13** produced previously (see first part) by amide formation mediated by BOP and $^iPr_2NEt$ in DMF, and subsequent protection of the two hydroxyls with TES group, to yield **17** (Figure 12).

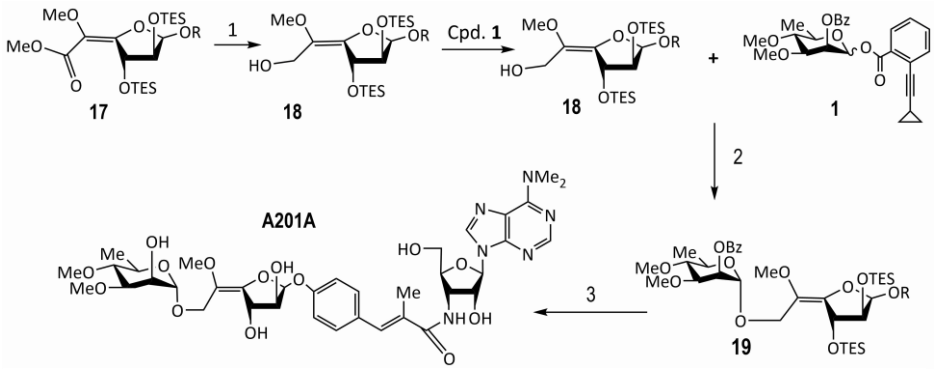


1.- BOP and $^iPr_2NEt$ in DMF at rt, 78%. 2.- TESOTf, Et_3N in CH_2Cl_2 at rt, 83%.

Figure 12. Preparation of **17** from **13** and **16**, by amide formation and hydroxyl protection.

In order to complete the synthesis of A201A, compound **17** was treated with $LiBH_4$ to reduce the terminal ester into alcohol **18**. The second glycosidic bond present in A201A was produced by condensation of alcohol **18** with ortho-alkynylbenzoate **1,** by carrying out the same procedure catalyzed by gold of the first glycosidation step (see above). In this case, a stoichiometric amount of the catalyst was needed to produce glycosydated **19**. Here, the basic properties of

the purine limited the protodeauration process that restored the catalyst for a new catalytic cycle (see Figure 10).[24] Finally, the α-rhamnoside **19** was converted into the antibiotic A201A by elimination of TES and benzoyl protective groups by treating with NaOMe in $MeOH/CH_2Cl_2$ (Figure 13).



1.- $LiBH_4$ in THF at -40ºC to rt, 78%. 2.- $Ph_3PAuOTf$ (1.0eq) in CH_2Cl_2 at 0 ºC, 55%.

3.- NaOMe in $MeOH/CH_2Cl_2$ at rt, 76%.

Figure 13. Completion of **A201A** synthesis, by glycosidation between **18** and **1** catalyzed by Au(I) and deprotection.

6.2. Biosynthesis

The high structural similarities with Hygromycin A and Puromycin clearly suggest that A201A is a hybrid metabolite of these two antibiotics, resulting from the combination of their two biosynthetic routes. For this reason, it is not surprising that genes that code the biosynthesis of Hygromycin A[25-27] and Puromycin[28, 29] were also found in the gene cluster that operates in A201A. One proof of the evolutionary relationships between their biochemical machineries, the three antibiotics coincide in having an acid moiety derived from coumaric acid, (Figure 14, in violet).

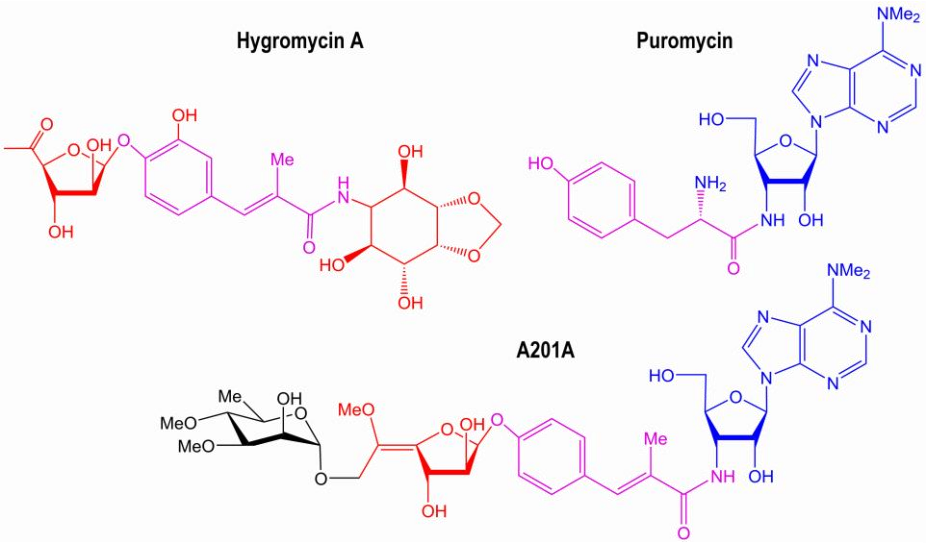


Figure 14. Similarities between the structures of **Hygromycin A**, **puromycin** and **A201A**.

The biosynthesis of the antibiotic A201A was independently elucidated by two different groups. Fernández-Lobato and col.[30, 31] who studied the production in *Saccharothrix mutabilis* subsp. *capreolus* and Ju and col.[8, 32] who did it in *Marinactionospora thermotolerans* deep-sea (Mtd). The two bacterial species essentially showed the same organization of biosynthetic genes with very small differences. Given the similarity between the two biosynthetic routes, here it will be only reviewed the proposal of the Ju's group[32] as it provided a more structurally and mechanistically detailed pathway, and also analyzed the formation of the metabolic intermediates and characterized their structures.

Below in figure 15, it is depicted the cluster of genes of A201A that was determined by correlation with genes producing puromycin and hygromycin. In comparison with these two antibiotics, an additional cluster of genes was found that probably accounted for the structural variances in A201A. The functionality of each gene was essentially deduced by homology of sequences via GenBank database and through the generation of enzymatic mutants producing fragmented metabolites.

Figure 15. Organization of the **A201A** biosynthetic gene cluster in *Marinactionspora thermotolerans* deep-sea (Mtd) according to ref. 32.

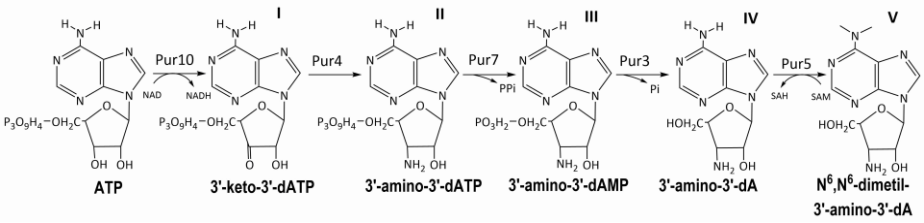
It was shown that the cluster genes operative in the A201A biosynthesis were MtdE through $MtdM_4$ (Mtd refers to the bacteria and each letter represents a gene) and, according to the authors, comprised all the genes necessary for the completion of the metabolite. Within the biosynthetic genes, as shown in figure 15, the MtdE, F, G, and M_1 genes were homologous to those of the puromycin pathway and participated in the biosynthesis of *N,N*-dimethyl-3'-amino-3'-deoxyadenosine, whereas the MtdI, N, O, S, T, U, and V genes were homologous to those operating in hygromycin A and biosynthesized the moiety *p*-hydroxy-α-methylcinnamic acid.

The elucidation of biosynthetic pathway showed that the bacterium produced the antibiotic in a specific order: first, the aminonucleoside moiety (Figure 14, moiety in blue), followed by the cinnamate moiety (Fig. 12, moiety in violet) and ending with the disaccharide fragment (Fig. 14, moieties in black and red). The biosynthesis of each moiety is discussed below.

6.2.1. Steps related biosynthesis of the aminonucleoside moiety

The enzymes that participated in this biosynthesis were MtdE, F, G and M_1 and were highly similar to Pur3, Pur4, Pur5, Pur7, and Pur10 that operated in the puromycin biosynthesis.[28, 31] After a series of experiments and comparisons by sequence homology, it was proposed that the biosynthetic pathway started from ATP, as occurred in puromycin (see scheme 1).

It was believed that adenosine entered the pathway through ATP by a series of enzymatic steps. Pur10, a NAD-dependent ATP dehydrogenase, converted adenosine into 3'-keto-3'-deoxy-ATP (**I**). This intermediate was probably modified by aminotransferase Pur4 into 3'-amino-3'-deoxy-ATP (**II**). As this intermediate was a strong inhibitor of RNA polymerase, it was probably detoxified by a pyrophosphatase Pur7, producing 3'-amino-3'-deoxy-AMP (**III**), but it was not clear whether Pur4 or Pur7 acted first. Then, the intermediate might be dephosphorylated by the monophosphatase Pur3 to give 3'-amino-3'-deoxy-A (**IV**). It was probably followed by the double methylation of nitrogen-6 by Pur5, a SAM-dependent methyltransferase, to yield N^6,N^6-dimethyl-3'-amino-3'-deoxyadenosine (**V**).



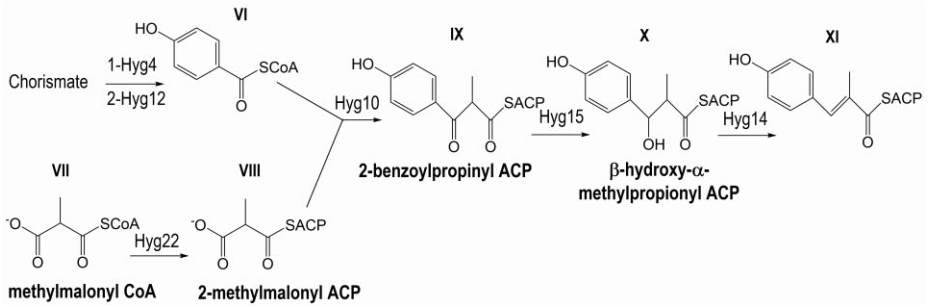
Scheme 1. Biosynthesis of the aminonucleoside moiety.

6.2.2. Steps involved in the biosynthesis of the cinnamate moiety

The authors indicated through sequence homology that the MtdI, N, O, S, T, U, and V enzymes involved in A201A biosynthesis were most probably homologous to those involved in the hygromycin A biosynthesis, namely, Hyg4, 10, 12, 14, 15, and 22.[27]

The pathway to this moiety was proposed to start with *p*-hydroxybenzoic acid (scheme 2), which derived from chorismate, a precursor produced by the shikimate pathway. Chorismate was first transformed by a putative chorismate lyase, encoded by Hyg4 = MtdV,[33] to yield *p*-hydroxybenzoic acid. In the next step, the *p*-hydroxybenzoic acid was probably activated by conversion to a thioester (**VI**), catalyzed by a CoA-ligase, Hyg12. In parallel, a putative acyltransferase Hyg22 catalyzed the conversion of methylmalonyl CoA (**VII**) to 2-methylmalonyl ACP (**VIII**).

Then, a decarboxylative condensation occurred between the active acid and the methylmalonyl ACP that could be catalyzed by the Hyg10, a ketoacyl synthase, to yield 2-benzoylpropionyl ACP (**IX**). A subsequent reduction of the keto group into hydroxyl was produced by the Hyg15, a 3-ketoacyl ACP reductase, to produce a β-hydroxy-α-methylpropionyl ACP (**X**). The biosynthesis was ended by dehydration, catalyzed by the Hyg14, a 3-hydroxyacyl ACP dehydratase, to produce the cinnamyl ACP moiety (**XI**).

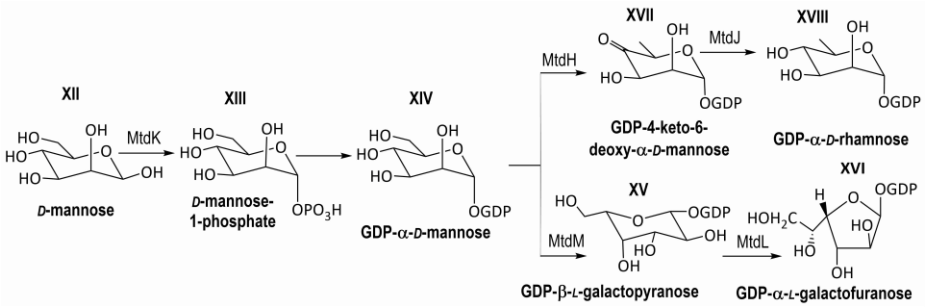


Scheme 2. Biosynthesis of the cinnamate moiety.

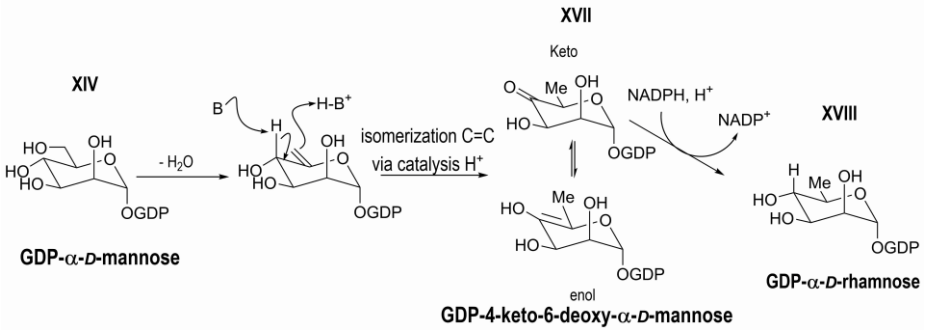
6.2.3. Steps involved in the biosynthesis of the disaccharide fragment

Experiments performed by the Ju's group, suggested that the two sugar units were derived from the same precursor, GDP-D-mannose. To confirm this hypothesis, D-mannose (**XII**) was marked with ^{13}C by fermentation in a *M. thermotolerans* strain. When it was analyzed by ^{13}C NMR, it was clearly seen that the two anomeric carbons of the two sugars units were enriched demonstrating that the two sugars used GDP-D-mannose as a biosynthetic precursor (see scheme 3).

Therefore, the biosynthetic pathway of the sugar moieties in A201A was started from the phosphorylated D-mannose (**XIII**) catalyzed by MtdK. Then, by the action of a GDP-sugar pyrophosphorylase, **XIII** was converted into GDP-α-D-mannose (**XIV**). This intermediate was then first processed by MtdM, a GDP-α-D-mannose 3',5'-epimerase, to produce GDP-β-galactopyranose (**XV**) by epimerization of 3' and 5' positions, and subsequently isomerized into GDP-α-D-galactofuranose (**XVI**) by a pyranose-furanose mutase, MtdL. In parallel, GDP-α-D-mannose was also reduced to GDP-α-D-rhamnose (**XVIII**) by the consecutive action of the MtdH and MtdJ enzymes via 4',5'-dehydration and enol-keto isomerization to GDP-4-keto-6-deoxy-D-mannose (**XVII**), followed by the reduction of 4-keto group into 4-hydroxyl. The structural basis of GDP-α-D-mannose conversion into GDP-α-D-rhamnose in two enzymatic steps (scheme 4) had been previously studied by Lam et al. in *Pseudomonas aeruginasa*.[34]



Scheme 3. Biosynthesis of the disaccharide moieties
GDP-α-L-galactofuranose (**XVI**) and GDP-α-D-rhamnose (**XVIII**).

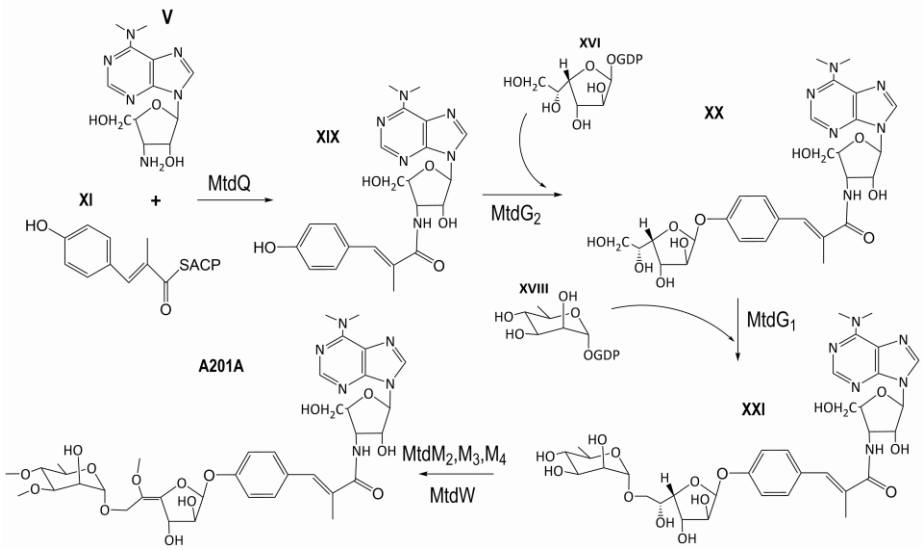


Scheme 4. Conversion of GDP-α-D-mannose to GDP-α-D-rhamnose according to ref. 34.

6.2.4. Steps involved in the coupling of moieties and additional methylations

MtdQ was a ligase homologous to Hyg12 that was responsible for the coupling of deoxyadenosine and cinnamyl moiety by forming an amide bond (**XIX**). It was believed that two glycotransferases acted consecutively, $MtdG_2$ that was responsible for the transfer of the hexafuranose moiety (**XX**), and $MtdG_1$ for the rhamnose moiety (**XXI**).

This biosynthesis was completed by action of three regiospecific methyltransferases and a desaturase. The methylases catalyzed *O*-methylations of glycosidic units, two of which acting on the rhamnose moiety, $MtdM_2$ responsible for the *O*-methylation of OH-C_4 and $MtdM_3$ of OH-C_3, and $MtdM_4$ was believed to catalyze *O*-methylation of the OH-C_5 of the GDP-α-L-galactofuranose unit. The biosynthesis of A201A was completed according to the authors by the desaturase MtdW that catalyzed a dehydrogenation of C_4-C_5 bond of the GDP-α-L-galactofuranose moiety.



Scheme 5. Completion of the A201A biosynthesis by consecutive transfer of the moieties regiospecific methylations and dehydrogenation.

Further studies revealed that if MtdA protein was mutated, and thus inactivated, A201A was notably produced with a yield 25-fold higher than in the wild type.[8]

6.2.5. Steps involved in the self resistance against A201A

Organisms that produce potentially autotoxic antibiotics possess mechanisms for self-resistance.[35] These mechanisms include objective modification, drug inactivation or an antibiotic outflow system. In some cases, they may have more than one of the mechanisms. Moreover, antibiotics should have devices for exporting the drug or its inactivated form. Membrane systems involved in secretion and/or resistance to certain antibiotics have also been found.

The biosynthetic proposal of A201A by Fernández-Lobato's group explained above suggested that an additional protein Ata9 was produced in *Saccharothrix mutabilis* that could be involved in providing self-resistance to A201A. This protein proved to be a transmembrane protein, which would be probably involved in the A201A outflow.[30]

The Barrasa's group reported the existence of an enzyme named Ard1 that participated in self-resistance to A201A.[36] It belongs to a family of membrane transport proteins dependent on ATP which produce active transport of A201A. The resistance conferred by Ard1 appeared to be

highly specific for A201A and might also be responsible for the exclusion of any enzymatically inactivated form of A201A.

After the discovery of Ard1, the Barrasa's group was able to encode the enzyme Ard2 which showed phosphotransferase activity on A201A.[37] The resulting phosphorylated product P-A201A was totally inactive. This represented a second specific resistance mechanism for A201A in addition to the ATP-dependent exclusion system caused by Ard1.

The phosphorylation by Ard2 took place on the 2'-hydroxyl group of the unsaturated hexafuranose moiety of A201A (Fig. 16). The site of phosphorylation was determined by comparison of the ^{1}H-NMR spectra of the original molecule with the phosphorylated one. Specifically, the phosphorylation was revealed by an increase of the chemical shift of the proton H2', and a widening of the signal due to coupling with phosphorus.

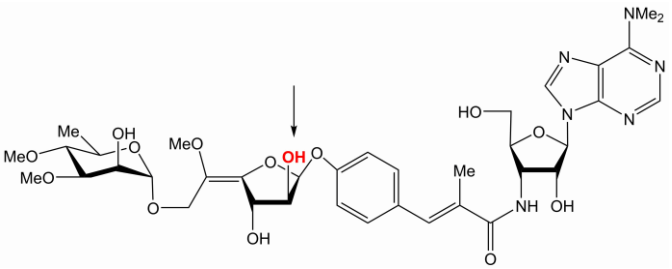


Figure 16. The site of phosphorylation of A201A produced by Ard2 indicated by an arrow.

What it could not be known was at which point the phosphorylation took place on A201A. As the bacterial ribosome is sensitive to A201A, the modification of the antibiotic probably occurred after its biosynthesis. It was presumed that A201A was reactivated during or after cell exclusion by specific hydrolases that eliminated the phosphate group.

6.3. Antibacterial activity of A201A and its analogs

In one of the experiments performed by the Ju's group,[33] the MtdV enzyme was mutated to know if it was a chorismate lyase (see above). As a result, the resulting mutant enzyme did not produce 4-hydroxybenzoic acid (4HB). It was envisaged that feeding thus mutated strain with halo-4HB, halogenated analogs of A201A could be obtained. On this basis, 3-F-4HB, 3-Cl-4HB, 3-Br-4HB, and 3-I-4HB were assayed as building blocks (Fig. 17). What was found is that only

those precursors containing F and Cl led to the production of new compounds, namely 3'-F- and 3'-Cl-A201A. Seeing the opportunity that novel compounds could be produced in this way, other 4HB precursors were additionally tested. After a series of feeding experiments, 3-methyl-4HB, 3-amino-4HB, 3-hydroxy-4HB, 2-F-4HB, and 2-Cl-HB containing analogues were obtained (Fig. 17). Consequently, these results demonstrated that the biosynthetic machinery of A201A was flexible enough to accommodate various 4HB as building blocks to produce structurally varied analogues of A201A for structure-activity relationship studies.

The antibacterial properties of all these A201A analogs were subsequently assayed. They showed to be inactive against a Gram-negative bacteria as *E. coli.* Most of the analogs were also inactive against Gram-positive bacteria (*Bacillus subtilis*, *Bacillius thurgienis*, *Micrococcus luteus*, *Straphylococcus aureus*), with the exception of 3'-F-A201A and 3'-Cl-A201A which were as active as A201A (MIC 1-8 μg mL^{-1}).

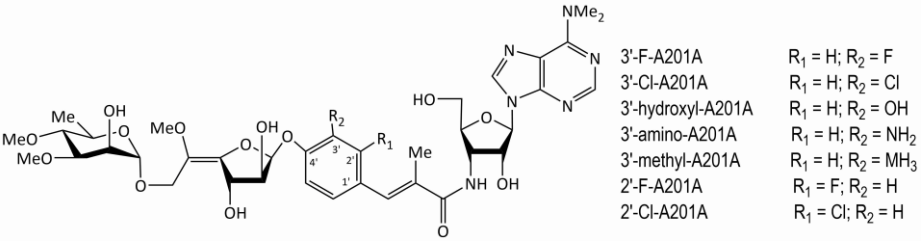


Figure 17. The structure of new A201A analogs obtained from feeding experiments with various 4HB building blocks.[32]

The Ju's group also experimented the production of A201A analogs by mutation of methylases and desaturase.[32] The novel compounds that were produced are depicted in Figure 18.

Here, the antibacterial activity of the resulting analogs was also compared with respect to A201A. Notably, it was found that non-*N,N*-dimethylated adenine compound showed the same antibacterial activity as A201A. Conversely, the elimination of the other methyl groups on the two terminal glycosides decreased antibacterial activity by half. The elimination of the terminal sugar or the saturation of the double bond within hexafuranose also decreased significantly the bioactivity.

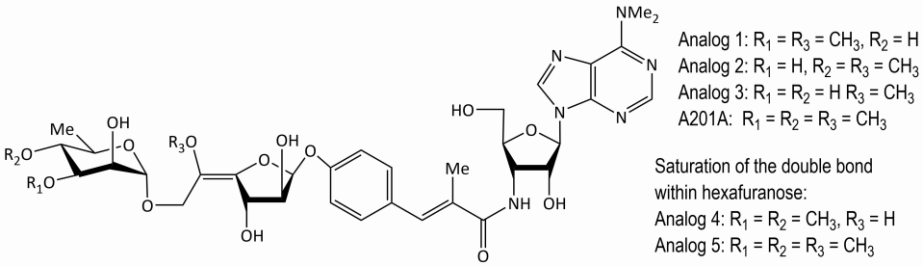


Figure 18. The structure of new A201A analogs obtained by mutation of methylases and desaturase.[32]

7. CONCLUSIONS

A201A, a complex aminonucleoside formed by an adenosine linked to two glycosyl units and coumarate, is a new antibacterial agent against aerobic and anaerobic gram-positive bacteria, as well as anaerobic gram-negative bacteria. It was found act by binding to the A-site large ribosome subunit and inhibiting the formation of the peptide bond.

A total chemical synthesis of A201A was reported by starting from cheap and simple raw materials by 47-steps convergent process. Notably, the use of a gold(I) catalyst allowed smoother and more suitable conditions for the formation of the two glycosidic bonds present in A201A.

It was shown that different bacteria biosynthesized A201A completely from a gene cluster. These bacteria also contained mechanisms of self-resistance to the antibiotic. These facts permitted to devise an efficient biosynthetic route for producing A201A and derivatives, and envisaged an alternative mode of action of antibiotics against the mechanisms of self-resistance in A201A producing bacteria.

A mutant enzyme permitted to prepare chemically varied A201A analogs, and perform structural-antibacterial activity studies. Some of these analogs showed similar activity to A201A, so new antibiotics with greater efficacy could be obtained in this way in the near future.

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9. ABBREVIATIONS AND ACRONYMS

A-site	Ribosome Aminoacyl-site
Aa-tRNA	Aminoacyl-tRNA
Ac	Acetyl
ACP	Acyl carrier protein
AMP	Adenosine monophospate
ATP	Adenosine triphosphate
BOP	Benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate
Bz	Benzoyl
CAN	Ceric ammonium nitrate
CoA	Coenzyme A
dA	Deoxyadenosine
dAMP	DeoxyAMP
dATP	DeoxyATP
DCM	Dichloromethane
DIAD	Diisopropyl azodicarboxylate
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
E. coli	*Escherichia coli*
E-site	Ribosome Exit-site
GDP	Guanosine diphosphate
HB	4-hydroxybenzoic acid
Hyg	Hygromycin
IBX	2-iodoxybenzoic acid
^{i}Pr	Isopropyl
MP	4-methoxyphenyl

MPOH	4-methoxyphenol
mRNA	Messenger ribonucleic acid
Mtd	*Marinactionospora thermotolerans* deep-sea
MTrCl	4,4'-dimethoxytriphenylmethyl chloride
NAD	Nicotinamide adenine dinucleotide
^{n}Bu	n-butyl
NMR	Nuclear magnetic resonance
NTf_2	Bis(trifluoromethane)sulfonimide (Bistriflimide)
P-site	Ribosome Peptidyl-site
P-tRNA	Peptidyl-tRNA
PTC	Peptidyltransferase center
Pur	Puromycin
Pyr	Pyridine
RNA	Ribonucleic acid
SAM	S-adenosylmethionine
SE	Trimethylsilylethyl
tRNA	Transfer ribonucleic acid
Tf	Trifluoromethanesulfonyl (triflyl)
TIPS	Triisopropylsilylether
TASF	Tris(dimethylamino)sulfonium difluorotrimethylsilicate
TMS	Tetramethylsilane
THF	Tetrahydrofuran
TES	Triethylsilyl ether
TESOTf	Triethylsilyl trifluoromethanesulfonate

APPENDICES

APPENDIX 1: ^{1}H-NMR A201A

Table 1. ^{1}H-NMR characterization data of A201A according to ref. 7..

		A201A 1H NMR signal (δ, ppm)*
Adenine moiety	H2	8.46 (s)
	H8	8.24 (s)
	CH_3	3.47(s)
Aminopentanose moiety	H1	6.05 (d, 2.5Hz)
	H2	4.56 (m)
	2-OH	5.95 (d, 4.5Hz)
	H3	4.60 (m)
	3-NH	7.83 (d, 7Hz)
	H4	4.22 (m)
	H5	3.59, 3.77 (d, 13Hz)
	5-OH	5.22 (d, 5Hz)
Aromatic acid moiety	Me	2.05 (d, 1Hz)
	Vinyl	7.27 (d, 1Hz)
	H2, H6	7.08, 7.17
	H3, H5	7.27, 7.36
Hexafuranose moiety	H1	5.86 (d, 4Hz)
	H2	4.10 (m)
	2-OH	5.57 (d, 6.5Hz)
	H3	4.55 (m)
	3-OH	5.65 (7Hz)
	5-OMe	3.49 (s)
	H6	4.01, 4.32 (d, 12Hz)

		A201A
Rhamnose moiety	H1	4.68 (d, 2Hz)
	H2	3.84 (m)
	2-OH	4.85 (d, 4.5Hz)
	H3	3.22 (dd, 3, 9Hz)
	3-OMe	3.29 (s)
	H4	3.02 (dd, 9, 9)
	4-OMe	3.40 (s)
	H5	3.55 (m)
	Me	1.17 (d, 6Hz)

*in parentheses, multiplicity of the signal and values of coupling constants