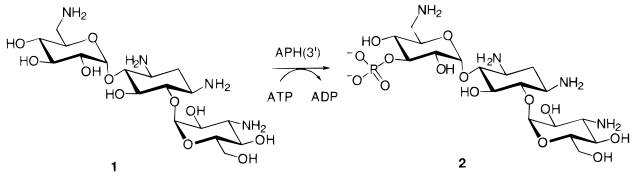
An Antibiotic Cloaked by Its Own Resistance Enzyme

Jalal Haddad, Sergei Vakulenko, and Shahriar Mobashery*

Department of Chemistry, Wayne State University
Detroit, Michigan 48202-3489

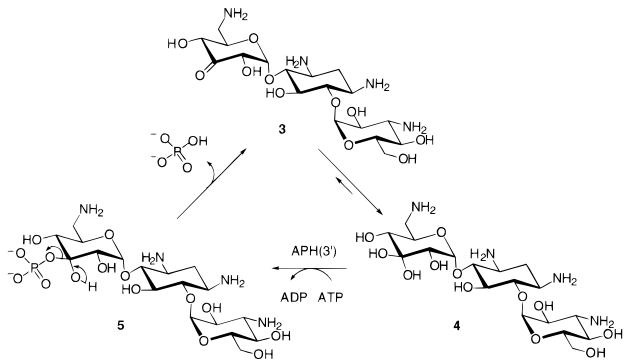
Received September 23, 1999

Discoveries of known bacterial pathogens that have become resistant to multiple antibiotics have been alarming in recent years,[1−11] stimulating renewed interest in development of novel antibiotics.[12−14] Microorganisms have evolved multiple mechanisms for resistance to antibiotics.[2,5,11,15] The most common mechanism for resistance to antibacterial agents is their structural modifications by resistance enzymes in the target organisms to inactive products. Three families of resistance enzymes for aminoglycoside antibiotics are known.[16] These are aminoglycoside phosphotransferases (APHs), aminoglycoside nucleotidyltransferases (ANTs), and aminoglycoside acetyltransferases (AAT).[16] Aminoglycoside 3′-phosphotransferases [APH(3′)s] are the most common and the best studied of these enzymes. The versatile aminoglycoside kanamycin A (**1**) was made clinically obsolete by the widespread expression of APH(3′)s in pathogens. These enzymes transfer the γ-phosphoryl group of ATP to the 3′-hydroxyl of aminoglycosides. The reaction of APH(3′)s with kanamycin A is depicted below (**1** → **2**).



1 **2**

It occurred to us that we could design a self-regenerating aminoglycoside that undergoes the enzymic phosphorylation at the 3′-hydroxyl, but chemically the structure of the phosphorylated molecule would be inherently *unstable*, leading to the elimination of phosphate and regeneration of the original antibiotic. We envisioned preparing an analogue of kanamycin A, compound **3**, for this purpose. Compound **3** would be in equilibrium with it hydrated variant **4**. We expected that compound **4** would experience phosphorylation by APH(3′)s, as these enzymes accept a broad range of molecules as substrates. The product of phosphorylation (**5**) would undergo the facile and spontaneous elimination of the phosphate moiety *nonenzymically*. Elimination of phosphate leads to the regeneration of **3**, which then recycles through the process.[17] If compound **3** were to have antibacterial activity, we would have designed/devised an aminoglycoside antibiotic that retains antibacterial property in the face of the APH(3′) activity, rendering these bacterial enzymes obsolete for the resistant bacterium. The phosphorylated antibiotic would be an intracellular reservoir for the active form of the drug, which is merely cloaked by the function of the resistance enzyme itself.



3 **5** **4**

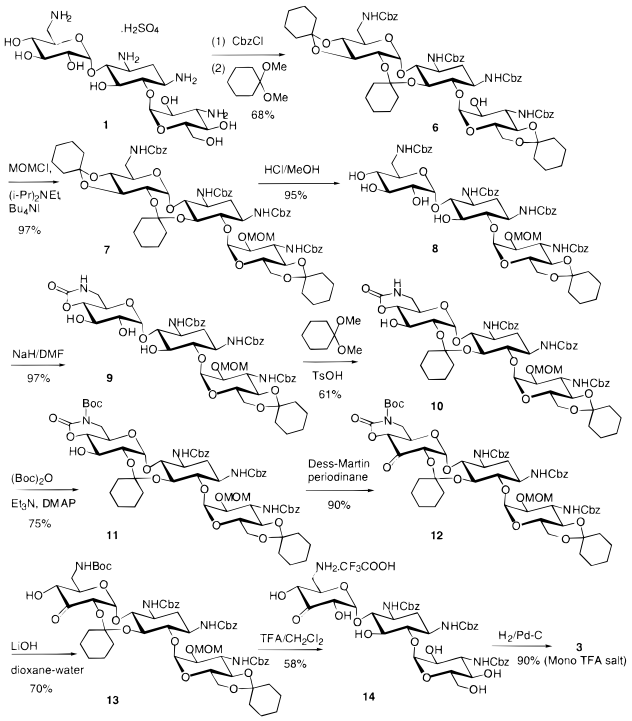
Scheme 1 shows the synthesis for compound **3** (details in the Supporting Information). The compound was obtained in 11 steps with an overall yield of 9%. The equilibrium for **3** and **4** favors the hydrated compound (1:4 mixture in aqueous solution), as assessed from the ^{1}H NMR of the sample in D_2O.

Compound **4** is indeed a substrate for both APH(3′)-Ia and APH(3′)-IIa (types Ia and IIa, respectively). The two enzymes transferred phosphate to **4** with the following kinetic parameters: $K_m = 5.7 \pm 0.7$ μM, $k_{cat} = 0.42 \pm 0.01$ s^{-1}, $k_{cat}/K_m = (7.0 \pm 1.0) \times 10^4$ M^{-1} s^{-1} and $K_m = 3.8 \pm 0.9$ μM, $k_{cat} = 0.11 \pm 0.01$ s^{-1}, $k_{cat}/K_m = (3.0 \pm 1.0) \times 10^4$ M^{-1} s^{-1}, respectively.[18] Once the antibiotic is phosphorylated, the product of the reaction releases the phosphate moiety. A spectrophotometric coupled assay was employed to link the consumption of ATP (due to phosphorylation of **4**) to oxidation of NADH by lactate dehydrogenase (Figure 1).[19] Compound **4** experienced phosphorylation and elimination of phosphate in the presence of APH(3′)-Ia, a process that repeated itself for at least eight times for each molecule of **3/4** in solution in 20 min of incubation, uninterrupted for as long as there was sufficient phosphoenolpyruvate (PEP) to facilitate recycling of ATP.[20] Upon depletion of NADH, additional amounts of the reagent would allow continuous monitoring of this ongoing process (again, until the ultimate depletion of PEP; Figure 1B).

(1) Cohen, M. A. *Science* **1992**, *257*, 1050.
(2) Davies, J. *Science* **1994**, *264*, 375.
(3) Oulette, M.; Kündig, C. *Int. J. Antimicrob. Agents* **1997**, *8*, 179.
(4) Amábile-Cuevas, C. F. *Antibiotic Resistance*: *From Molecular Basics to Therapeutic Options*; Chapman & Hall: New York, 1996.
(5) Chadwick, D. J.; Goode, J. *Antibiotic Resistance: Origins, Evolution, Selection and Spread*; John Wiley & Sons: New York, 1997.
(6) Fisher, J. A. *The Plague Makers*; Simon & Suster: New York, 1994.
(7) Garrett, L. *The Coming Plague*: *Newly Emerging Diseases in a World out of Blance*; Farrar, Straus, and Giroux Publishing: New York, 1994.
(8) Harrison, P. F.; Ledenberg, J. *Antimicrobial Resistance: Issues and Options*; National Academy Press: Washington, DC, 1998.
(9) Jungkind, D. L.; Mortensen, J. E.; Fraimow, H. S.; Calandra, GB *Antimicrobial Resistance: A Crisis in Health Care*; Plenum Press: New York, 1995.
(10) Levy, S. B. *The Antibiotic Paradox: How Miracle Drugs Are Destroying the Miracle*; Plenum Press: New York, 1992.
(11) Rosen, B. P.; Mobashery, S. *Resolving the Antibiotics Paradox*; Plenum Press: New York, 1998.
(12) Chu, D. T. W.; Plattner, J. J.; Katz, L. *J. Med. Chem.* **1996**, *39*, 3853.
(13) Krantz, A. *Nature Biotech.* **1998**, *16*, 1294.
(14) Vetter, D. *Drug Discov. Today* **1998**, *3*, 404.
(15) Mobashery, S.; Azucena, E. *Mechanisms of Bacterial Antibiotic Resistance*; *Encyclopedia Life Science*; Macmillan Reference Ltd: Basingstoke, UK, 1999; in press.
(16) Wright, G. D.; Berghuis, A. M.; Mobashery, S. *Aminoglycoside Antibiotics: Structures, Functions and Resistance*; Rosen, B. P., Mobashery, S., Eds.; Plenum Press: New York, 1998; pp 27−69.
(17) Two publications (Hayashi, S. I.; Lin, E. C. C. *J. Biol. Chem.* **1967**, *242*, 1030. Rendina, A. R.; Cleland, W. W. *Biochemistry* **1984**, *23*, 5157) have shown that glycerol kinase, fructose-6-phosphate kinase, fructokinase, and hexokinase accept aldehydes and ketones as substrates. Phosphorylation of the hydrated carbonyl is followed by the release of phosphate in these cases as well.
(18) These parameters are comparable for those for phosphorylation of the parent compound kanamycin A (**1**): $K_m = 2.8 \pm 0.3$ μM, $k_{cat} = 1.7 \pm 0.1$ s^{-1}, $k_{cat}/K_m = (6.0 \pm 0.8) \times 10^5$ M^{-1} s^{-1} and $K_m = 3.1 \pm 0.5$ μM, $k_{cat} = 0.65 \pm 0.02$ s^{-1}, $k_{cat}/K_m = (2.1 \pm 0.4) \times 10^5$ M^{-1} s^{-1} for APH(3′)-Ia and APH(3′)-IIa, respectively.
(19) Siregar, J. J.; Lerner, S. A.; Mobashery, S. *Antimicrob. Agents Chemother.* **1994**, *38*, 641.
(20) A typical assay mixture contained 200 mM PIPES buffer (pH 7.4), 11 mM magnesium acetate, 22 mM potassium acetate, 1.8 mM PEP, 0.1 mM NADH, 6.1 units of PK, 21 units of LD, 1 μM APH(3′)-Ia, 10 μM compound **3**, and 0.15 mM ATP. The final volume for each assay mixture was 1.0 mL.

10.1021/ja993452o CCC: $18.00
Published on Web 12/04/1999

Scheme 1



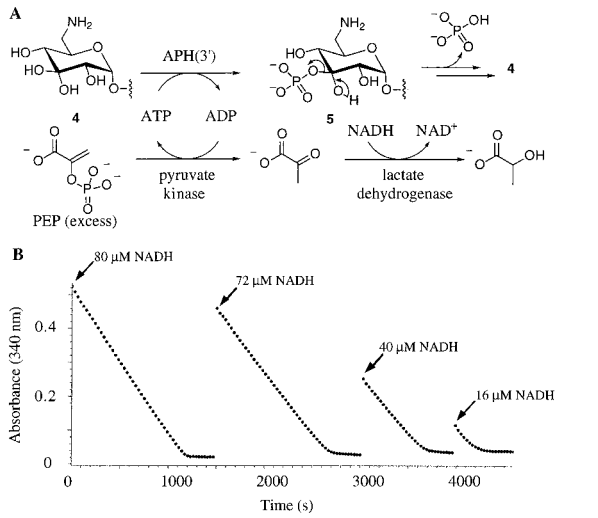


Figure 1. (A) The coupled spectrophotometric assay of pyruvate kinase and lactate dehydrogenase recycles ATP, which is consumed by the APH(3′)-mediated phosphorylation of **4**. (B) The gradual disappearance of the chromophore for NADH, as a function of phosphorylation of **4**, was monitored by the decline in absorbance at 340 nm. The process is continuous until PEP (180-fold excess) is entirely depleted (details in ref 20). Each run was initiated/reinitiated by the addition of the given quantity of NADH to the recycling mixture.

We also attempted to correlate consumption of ATP with the release of inorganic phosphate from the antibiotic. Within 3 min of incubation of the recycling mixture, we measured 12.5 ± 1.2 μM of ATP consumed (concentration of the antibiotic in the assay mixture was 10 μM). The Malachite Green assay for inorganic phosphate[21] after 3 min of incubation gave 11.03 ± 0.09 μM of inorganic phosphate. Hence, consumption of ATP and release of inorganic phosphate appear to proceed one after another (the measured concentration of inorganic phosphate after 20 min of incubation was about 80 μM for the 10 μM of antibiotic in solution). The same mixture in the absence of compound **3** gave a negligible value for the content of free inorganic phosphate.

Table 1. Minimum Inhibitory Concentrations (MICs) for Compound **3** and Kanamycin A against *E. Coli* JM83 and Its Engineered Isogenic Variants

	MIC (μg/mL)	
strains[a]	kanamycin A	compound **3**
E. coli JM83 pACYC177	4000	1000
E. coli JM83 pUC19::Km	8000	1000
E. coli JM83	8	250

[a] Preparation of the strains are described in the Supporting Information.

Table 1 summarizes the biological activities of kanamycin A and compound **3** against a set of isogenic laboratory strains. *Escherichia coli* JM83, used in these experiments, is a laboratory variant of the strain K-12. Compound **3** is not as active as kanamycin A against this laboratory microorganism. However, we opted to use this strain since its aminoglycoside resistant variants have been engineered in our laboratory. The following analysis demonstrates that compound **3**, as the prototype molecule of its kind, performs in vivo as was intended that it should. The gene for APH(3′)-Ia was engineered into a low-copy plasmid in *E. coli* JM83 pACYC177 and in a high-copy plasmid in strain *E. coli* JM83 pUC19::Km. The high-copy and low-copy bacterial strains increased the minimum inhibitory concentration (MIC) for kanamycin A by 1000- and 500-fold, respectively. The same two strains increased the MIC for compound **3** by a mere 4-fold.

Hence, this type of antibiotic does not attempt to circumvent the resistance enzymes,[22] but it is simply not affected appreciably by their catalytic function. Not only would this antibiotic make the presence of the resistance enzymes obsolete in the clinical strains, the potential future use of this type of antibiotic in the clinic might be an impetus for the organisms that harbor the plasmid-borne genes for these enzymes to lose them (i.e., reverse selection).

Acknowledgment. This work was supported by a grant from the National Institutes of Health. We are indebted to David Lawrence for bringing to our attention citations given under footnote 17. High-resolution mass spectral data were obtained at the Michigan State University Mass Spectrometry Facility, which is supported, in part, by a grant (DRR-00480) from the Biotechnology Research Technology Program, National Center for Research Resources, and National Institutes of Health.

Supporting Information Available: Detailed procedures for syntheses and kinetic determinations (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA993452O

(21) Baykov, A. A.; Evtushenko, O. A.; Avaeva, S. M. *Anal. Biochem.* **1988**, *171*, 266.

(22) Antibiotics such as dibekacin and tobramycin lack the 3′-hydroxyl group and antibiotics isepamycin and amikacin have certain acyl moieties at the N-1 position that make them poorer substrates for the phosphotransferases. These molecules attempt to circumvent the resistance enzymes.