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A Chemoenzymatic Route to Diversify Aminolgycosides Enables a Microarray-Based Method to Probe Acetyltransferase Activity

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This worked is dedicated to Professor Peter H. Seeberger in honor of his receiving the Tetrahedron Young Investigator Award.

Specific modification of functional groups in aminoglycosides poses a significant synthetic challenge. A chemoenzymatic route for modification of aminoglycosides is disclosed. The critical feature of this approach is the discovery that the aminoglycoside 3-*N*-acetyltransferase, AAC(3)-IV, from *Escherichia coli*[1] accepts azidoacetyl coenzyme A (AzAcCoA) as a substrate in a similar manner as the natural substrate, acetyl coenzyme A (AcCoA). After enzymatic delivery of an azidoacetyl group, it can be chemically modified by a Huisgen dipolar cycloaddition reaction (HDCR),[2] therefore enabling further diversification. Thus, this method accelerates access to modified compounds with diversity beyond that which can be installed directly by AAC(3) and a modified CoA thioester. The approach was further developed to study modification of aminoglycosides with AAC(3), which causes broadscale aminoglycoside inactivation, by using a fluorescence-based microarray platform. This platform is a useful analytical tool for the facile identification of both protein and carbohydrate substrates for acetyltransferases, which play critical roles in a multitude of cellular processes.[3]

Aminoglycosides represent one of the largest classes of antibacterials with activity against both Gram-negative and Gram-positive bacteria. Many aminoglycosides exert their antibacterial activity by binding to the decoding site (A-site) in 16S rRNA.[4] Binding to this site affects recognition of cognate and noncognate tRNAs by the ribosome. As is common with all antibiotics, resistance to aminoglycosides has emerged since their initial introduction as therapeutic agents.[5] One of the most important resistance-causing mechanisms against aminoglycosides is enzymatic modification, for example, by acetyltransferases (AACs). Aminoglycosides have also been used to facilitate translational readthrough in diseases caused by nonsense mutations, including Duchenne's muscular dystrophy, cystic fibrosis, and hemophilia.[6] To better exploit these targets, simple access to modified aminoglycosides with improved biological activity, such as reduced susceptibility to modification by resistance-causing enzymes or more specific recognition of target RNAs, is needed. Synthesis of designer aminoglycosides, however, is difficult because they contain a variety of amino and hydroxy groups with similar reactivities.

In an effort to develop expedited routes that lead to site-specifically derivatized aminoglycosides, we studied the ability of AAC(3) to accept modified acetyl coenzyme A substrate to install functional groups in their structures that can be further chemically diversified. Towards this end, AzAcCoA was synthesized, and its ability to acylate the aminoglycoside apramycin was studied (Figure 1 A). AzAcCoA was used because the azido group can be diversified by reaction with alkynes using a copper(I)-catalyzed HDCR. Modification of apramycin by AzAcCoA was monitored by UV-vis[1] and mass spectrometry, and each study unambiguously showed that AzAcCoA is accepted as a substrate (Figure 1 B and C). In fact, AzAcCoA is accepted in a similar manner as the natural substrate. AzAcCoA has a K_M and a k_{cat} value that are fourfold and threefold lower, respectively, than AcCoA (Figure 1 B). The specificity constants (k_{cat}/K_M) are similar: $5.1 \times 10^5\,M^{-1}s^{-1}$ for AcCoA and $6.3 \times 10^5\,M^{-1}s^{-1}$ for AzAcCoA. In contrast, when propionyl CoA, malonyl CoA, and butyryl CoA substrates were studied, they had k_{cat}/K_M values that are decreased by 7-, 23-, and 1730-fold relative to AcCoA, respectively.[1] Another recent and extensive study has shown that a variety of aminoglycosides can be modified by CoA derivatives to provide new aminoglycosides. The k_{cat}/K_M of the modified CoA substrates in that study are also lower than AcCoA.[7] Thus, the similarity in specificity constants of AzAcCoA and AcCoA as substrates for AAC(3) points to unique features for AzAcCoA interacting with the enzyme.

Encouraged by the results of enzymatic azidoacetyl transfer, the scope of the 3-*N*-azidoacetyl-apramycin (**3**; Figure 1 A) modification by HDCR was further investigated. In initial studies, dansyl alkyne (**4**; Figure 1 A) was used to modify the azidoacetyl aminoglycosides (Figure 2). 3-*N*-Azidoacetyl-apramycin (**3**) was partially purified by passing the reaction mixture over Dowex ion-exchange resin (HO- form) to capture any CoA-containing materials. This material was then subjected to HDCR modification with compound **4**. Mass spectral analysis indicated complete conversion to **5.** The reaction was purified by HPLC, and **5** was obtained in 44% isolated yield over two steps.

Armed with these results, a microarray-based method to study the modification of 2-deoxystreptamine aminoglycosides by AAC(3) was developed. In these experiments, the extent of AAC(3) modification on the array surface was monitored by treating the generated azidoacetyl aminoglycosides with an alkyne dye through a HDCR (Figure 3 and 4).

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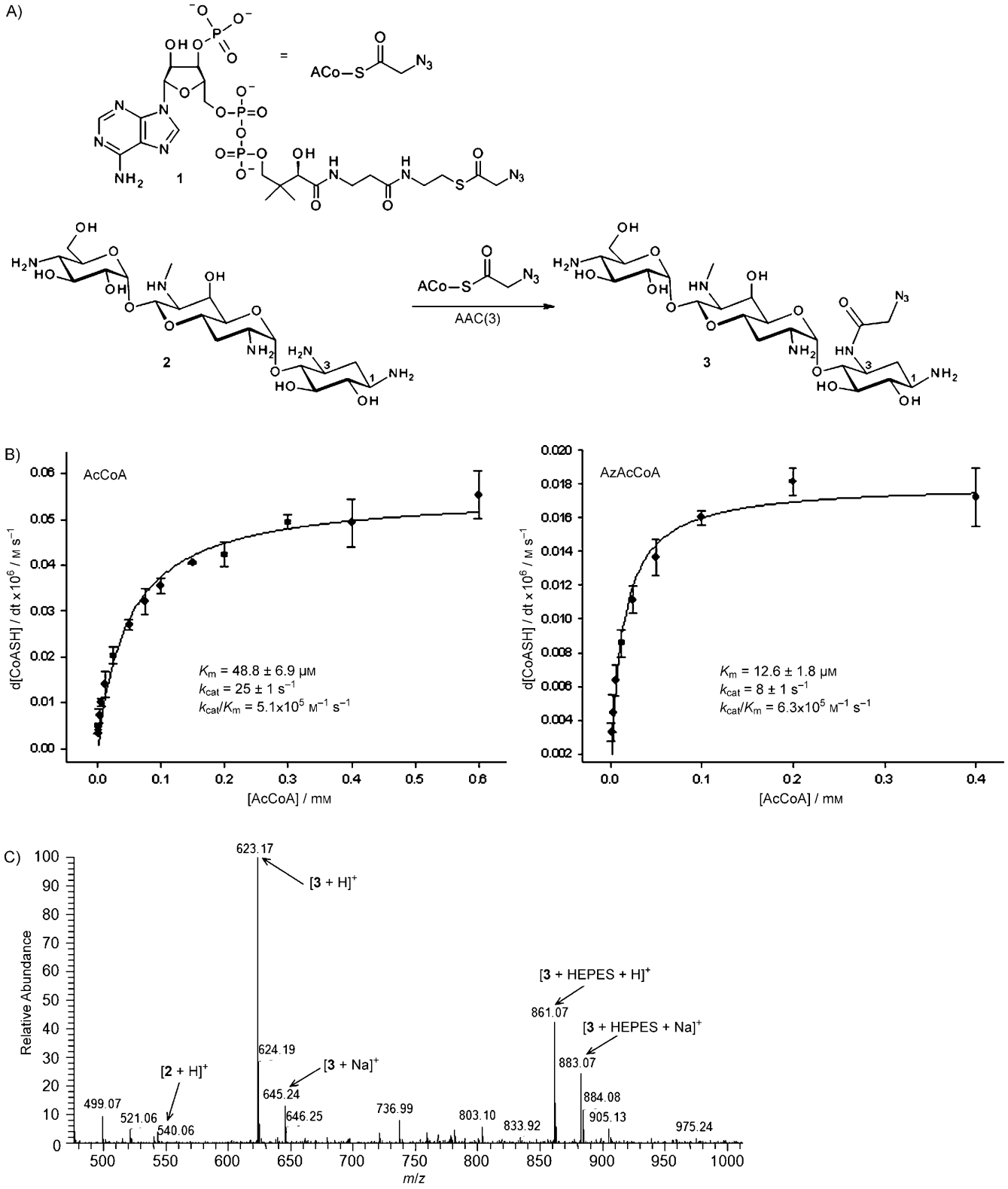

Figure 1. Studying the ability of AAC(3) to accept AzAcCoA as a substrate. A) Reaction of AzAcCoA with apramycin. B) Michaelis–Menten plots for the modification of apramycin by AcCoA and AzAcCoA. C) Mass spectral analysis of the reaction of apramycin with AzAcCoA.

In order to site-specifically immobilize the aminoglycosides onto a microarray, a small library of azide-modified aminoglycosides was synthesized (Figure 3). These compounds can be anchored onto alkyne-functionalized agarose microarrays.[8] Syntheses of compounds **6**–**14** were completed according to modification of previously published procedures in which a pri-

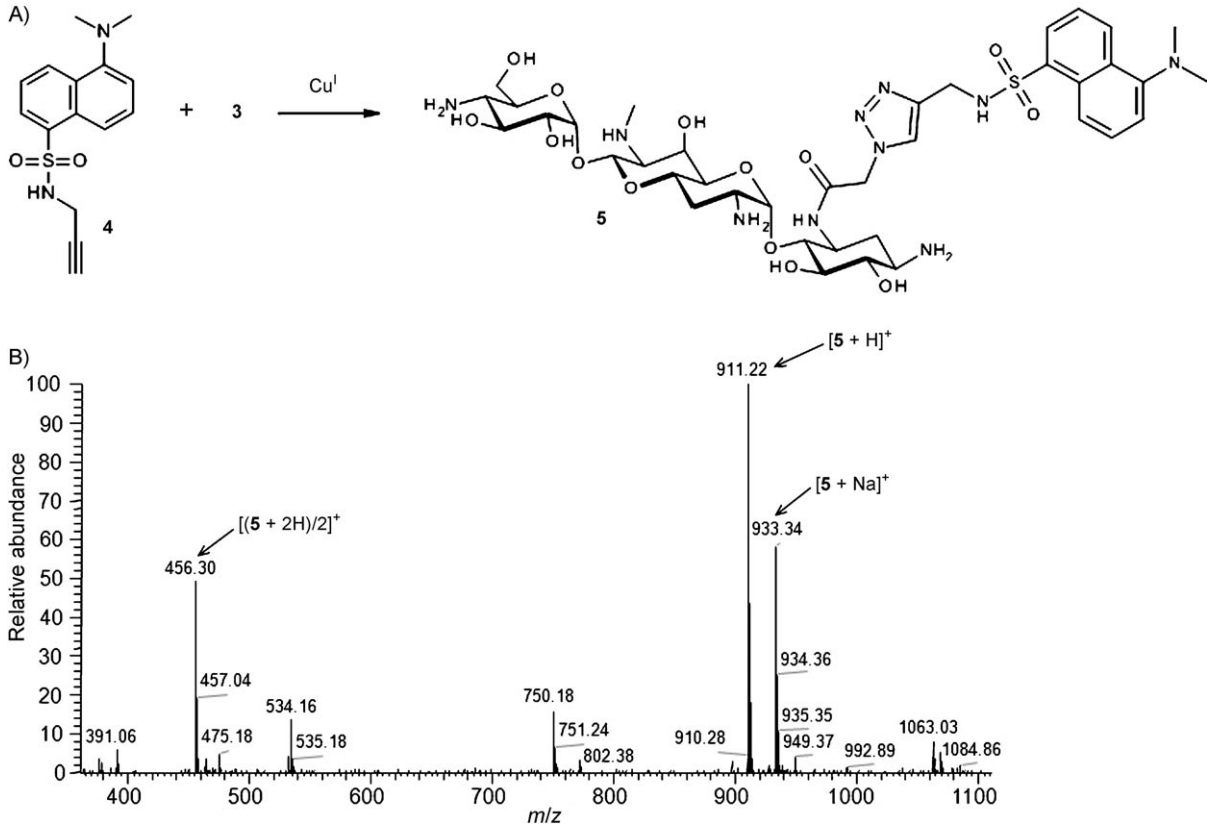

Figure 2. HDCR modification of 3-*N*-azidoacetyl-apramycin (**3**). A) Reaction between compounds **3** and **4**. B) Mass spectral analysis of the reaction to form **5**.

mary hydroxy group is activated by reaction with 2,4,6-triisopropylbenzenesulfonyl chloride. The resulting adduct is displaced with sodium azide.[8b,9] Compound **15** was synthesized by reductive amination of streptomycin with 3-azidopropylamine. Compound **16** was synthesized by acylation of spermine with 4-azidobutyric acid. Structures **6**–**14** are known to bind to the bacterial rRNA A-site,[10] and most have been studied as AAC(3) substrates.[1] Structures **15** (streptomycin derivative) and **16** (spermine derivative) are not known to be specific bacterial rRNA A-site binders. Compounds **15** and **16** are not expected to be substrates for AAC(3) because previous studies have shown that guanidiniylated aminoglycosides are not modified by acetyltransferases.[11]

The azido-aminoglycosides and spermine derivatives were arrayed onto alkyne-agarose microarrays in the presence of copper(I). Serial dilutions from 500 to 62 pmol of compound were delivered to the surface through a pin-transfer replicator. After the reaction to conjugate the ligands onto an array, the arrays were quenched[12] and then modified with AzAcCoA by AAC(3). After washing the arrays, they were treated with an alkyne-functionalized BODIPY dye via a HDCR (Figure 4A). As expected, scans of modified arrays showed clear spots of modification for **6**–**14**, whereas **15** and **16** were not labeled (Figure 4C and 4E). Array data for modification by AAC(3) were then compared to previous solution-based studies of modification.[1] In general, the two studies are in agreement. In solution-based studies, neomycin B has the highest k_{cat}/K_M value followed by tobramycin, paromomycin, apramycin, ribostamycin, kanamycin, and amikacin. The order of the extent of modification on the arrays at the highest spot loading is: neomycin B (**13**) > paromomycin (**6** and **7**) = amikacin (**9**) > kanamycin A (**12**) = neamine (**14**). Other aminoglycosides are modified to a lesser extent.

Arrays were also probed for binding to a fluorescently-labeled mimic of the bacterial rRNA A-site (Figure 4D). The data are in good agreement with a study of aminoglycoside binding to the A-site using surface plasmon resonance (SPR).[10] For example, neomycin B (**13**-like) gives the highest signal for binding to the A-site and is the highest affinity binder in the series (Figure 4F). The second and the third highest binding signals are observed for the paromomycin derivatives (**7** and **6**), and the apramycin (**10**) and tobramycin (**11**) derivatives, respectively. The other aminoglycosides that were expected to bind the A-site gave lower but measurable signals. The lowest binding signals are observed for the nonspecific binders, streptomycin (**15**) and spermine (**16**) derivatives, as expected.

Our fluorescence-based approach is advantageous over other microarray approaches that have been developed to probe aminoglycoside modification by resistance-causing enzymes that use radioisotopes. For example, detection of ^{14}C-labeled acetylated aminoglycosides requires several days of exposure in a phosphorimager cassette.[13] Furthermore, the reso-

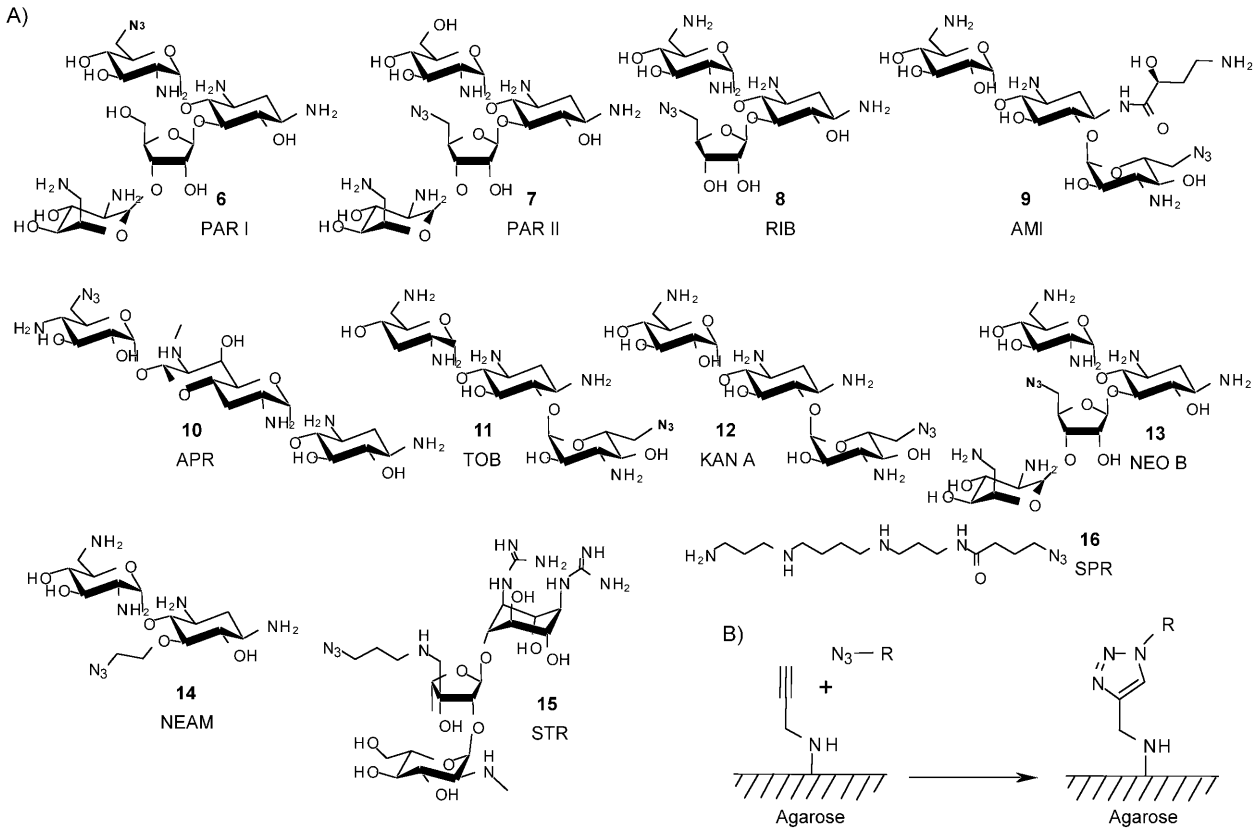

Figure 3. The compounds used to construct microarrays and the microarray immobilization chemistry used in this study. A) Structures of azide-functionalized aminoglycosides **6**–**15**, and spermine **16**. B) The HDCR used to anchor azide-containing small molecules onto alkyne-functionalized agarose microarray surfaces. (N_3-R represents azide-functionalized compounds **6**–**16**).

lution of a phosphorimager is significantly diminished relative to that of a fluorescent microarray scanner. Enhanced resolution allows for a significant increase in the number of features (spots) that can be analyzed on a microarray.

During the course of this work, a series of modified aminoglycosides were synthesized using modified coenzyme A derivatives.[7] Interestingly, these modified antibiotics displayed antibacterial activity despite the fact that they were modified by resistance-causing enzymes.[7] The results herein expand that work, demonstrating that aminoglycosides can be modified by coenzyme As that install reactive groups that can be easily chemically diversified. Thus, the diversity of modifications introduced into aminoglycosides is not solely limited by the ability of an AAC to accept a modified coenzyme A as a substrate. Rather, reactive tags can be installed on an aminoglycoside by a CoA derivative followed by subsequent chemical modification to provide access to novel compounds.

In summary, a chemoenzymatic route toward the synthesis of aminoglycosides is reported. This method was used to develop a high-throughput and sensitive microarray-based method to study the modification of aminoglycosides by acetyltransferases that confer resistance. These developments have broad applications in both the synthesis of diversified aminoglycosides and in developing microarray-based platforms to probe modification of biomolecules by acetyltransferases, including proteins and carbohydrates for which activities are modulated by acetyltransferases.[3]

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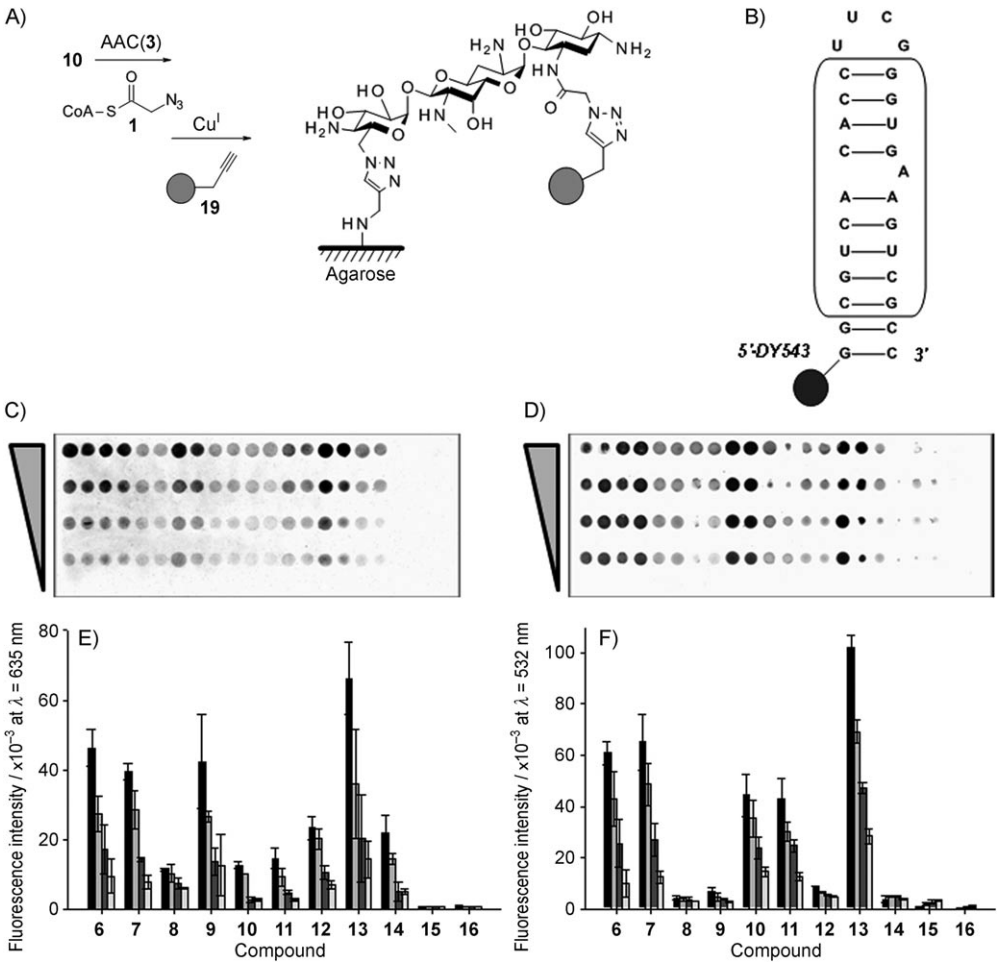

Figure 4. Probing aminoglycoside recognition by microarray. A) Labeling aminoglycosides by enzymatic delivery of an azidoacetyl group followed by installation of a dye by a HDCR. B) Secondary structure of the bacterial rRNA A-site mimic (boxed nucleotides represent those derived from the native sequence). C) Image of a microarray to study AAC(3) modification of aminoglycosides. D) Image of a microarray to study binding of an oligonucleotide mimic of the bacterial rRNA A-site. E) Representative plot of data for AAC(3) modification. F) Representative plot of data for binding of the rRNA A-site mimic. Each group of bars in the plots from left to right corresponds to delivery of 500, 250, 125, 62, and 0 pmol of material to the array surface.

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