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Designed Spiro-Bicyclic Analogues Targeting the Ribosomal Decoding Center

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The bacterial ribosome represents the confirmed biological target for many known antibiotics that interfere with bacterial protein synthesis. Aminoglycosides represent a lead paradigm in RNA molecular recognition and constitute ideal starting points for the design and synthesis of novel RNA binders. Previous rational design approaches of RNA-targeting small molecules have been mainly concentrated on direct functionalization of aminoglycosidic substructures. Herein, we successfully designed and synthesized rigid spirocyclic scaffolds locked in a predicted ribosome-bound "bioactive" conformation. These analogues are able to mimic many of the interactions of the natural products for the A-site, as proven by their obtained binding affinities. The development of an optimized approach for their synthesis and their potential to inhibit protein production in vitro are presented. Our results could be further utilized for the development of analogues with improved antibiotic profiles.

Introduction

The discovery of antibiotics, small molecules of natural and synthetic origin that specifically interfere with biological processes in bacteria, has been one of the major breakthroughs of medicine in the 20th century, allowing the treatment of life-threatening infectious diseases. Early after their discovery, the emergence of resistance mechanisms has been observed in bacteria, which counteract their action. The challenge of antibiotic resistance is particularly severe because most antibacterial drug classes used today have been in the clinic for more than three decades. The initial broad stream of novel antibiotics discovered from both natural and synthetic sources has been narrowed down to a trickle, with only two new classes of antibacterials, fluoroquinolones and oxazolidinones, developed over the last 30 years.[1] Novel potent antibiotics are thus required in addition to the currently used drugs. In many bacteria, including important pathogens, rrn operons that encode rRNA are present in multiple copies, thus rendering resistance development against ribosome-targeted antibiotics by mutations in the rRNA an unlikely event.[2]

The bacterial ribosome represents the confirmed biological target for many known antibiotics that interfere with bacterial protein synthesis.[3] Crystallographic analyses of ribosomal subunits and domains thereof in complex with antibiotics have demonstrated that the natural products interact almost exclusively with ribosomal RNA components (rRNA),[4] a result that lends support to earlier biochemical findings[5] and emphasizes the importance of RNA as a drug target.[6,7] Specifically for natural aminoglycosides, such as paromomycin, neomycin B, and neamine (Scheme 1), this is accomplished through their interaction with the decoding-site (A-site) ribosomal RNA[5,8] (Scheme 1) and disruption of the mRNA-decoding fidelity.[9,10] Upon binding, aminoglycosides displace two key adenine residues, which are involved in contacts with the mRNA–tRNA hybrid, from the deep groove of the decoding-site rRNA. The resulting conformational change leads to reduced discrimination against noncognate tRNAs and subsequently decreased translational accuracy. Despite their undesirable pharmacological profiles and resistance development limiting their antibiotic utility,[11] their capacity to bind with high affinity to the bacterial decoding site and several other RNA targets[12] renders them a lead paradigm in RNA molecular recognition.[13,14] Therefore, aminoglycosides and fragments thereof constitute ideal starting points for the design and synthesis of novel RNA binders.

Three-dimensional structures of aminoglycosides in complex with decoding-site oligonucleotides and whole 30S ribosomal subunits provide us important information regarding the molecular recognition principals of the decoding site by antibiotic ligands and the mechanics of translational fidelity.[10,15,16] Importantly, these studies underline a key role for the 2-deoxystreptamine (2-DOS, Scheme 1) moiety in RNA recognition.[17] In all cases, the cyclohexitol interacts with two consecutive base pairs of the decoding-site RNA, C1407–G1494 and the non-Watson–Crick pair U1406–U1495 (Scheme 1). Importantly, these base pairs are conserved in both the bacterial and eu-

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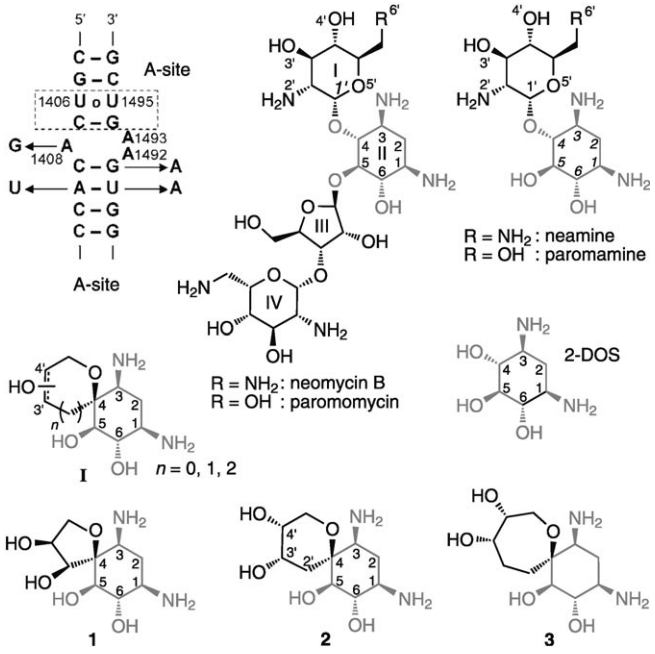


Scheme 1. Secondary structure of the bacterial decoding-site, depicting the internal loop (A-site) in 16S rRNA. The four base-changes of the eukaryotic sequence are indicated by arrows. The recognition site for the 2-DOS moiety of aminoglycosides is boxed; structures of neomycin B and paromomycin, neamine (neomycin A) and paromamine, 2-deoxy-streptamine (2-DOS) and the conformationally restricted analogues (general structure I, and specific constructs 1, 2 and 3) designed and synthesized in this study.

karyotic decoding sites. Examination of structurally characterized aminoglycoside complexes indicates that although the relative orientation of rings I and II (Scheme 1) is very similar, the conformation adopted by rings III and IV is significantly variable depending on the RNA target.[18] Consequently, the molecular discrimination observed in aminoglycosides for the bacterial target is accomplished by the strategic placement of sugar substituents at the 4- and 5- or the 4- and 6-positions of the 2-DOS moiety. The apparent rigidity of the neamine system (rings I/II, Scheme 1) underlines the importance of this module for RNA recognition because both rings (I and II, Scheme 1) contribute in key interactions that are responsible for target binding in decoding-site complexes with aminoglycosides.[18]

The target variability of the aminoglycosides has been attributed to two major factors: 1) Their highly charged nature, dictated by the number of amino-functionalities, which is responsible for the electrostatic nature of their RNA-binding mode and 2) their conformational adaptability, which provides limited flexibility and is responsible for the formation of well-defined drug complexes that are distinct from non-specific interactions of nucleic acids with flexible polyamines such as spermidine. The term *structural electrostatic complementarity* has been assigned to this promiscuous, yet target-specific binding of aminoglycosides to RNA.[13]

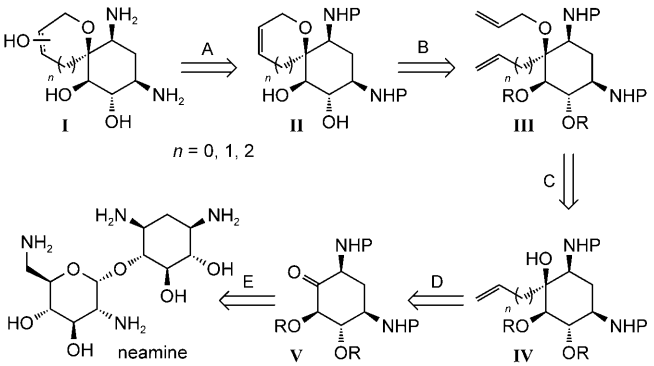
Rational design approaches of RNA-targeting small molecules have been previously reported, mainly concentrating on individually functionalized aminoglycosidic structural components, explicitly 2-deoxystreptamine (2-DOS)[19] and glucosamine,[20,21] as well as neamine[22,23] and paromamine.[24,25] Herein, by concentrating our efforts on the principles of *structural electrostatic complementarity*, we formulate an approach based on two major considerations. Firstly, the flexibility of the glycosidic bond in the natural products, although limited, might be responsible for the target variability demonstrated in their action. Specifically, comparison of the oligosaccharide conformations recognized by the bacterial ribosome and the enzymes responsible for the antibiotic inactivation, presents remarkable differences.[26] Thus, new chemical entities locked in the ribosome-bound "bioactive" conformation in which the glycosidic bond has been replaced by a rigid quaternary center, were targeted (**I**, Scheme 1).[27]

Secondly, the conservation of established contacts between natural aminoglycosides and the A-site RNA[28] represents a major priority of our design. Specifically, key characteristic interactions that we attempted to maintain (shown in Figure 1A for neamine) are: 1) The formation of a pseudo base pair with A1408; 2) The contacts with O4 of U1495; 3) The contact with N7 of G1494; and 4) The contacts with the phosphate group of A1492, A1493, and G1494. Consequently, small-molecule rigid scaffolds, like compound **2** (Scheme 1), were modeled in the bacterial A-site, exhibiting their oxa-spiro scaffold in the orientation shown in Figure 1C, relatively to neamine. Briefly, each designed compound was docked by using AUTODOCK 3[29] into an energy-minimized average model of the bacterial A-site, which was calculated from several crystallographic coordinates of RNA–aminoglycoside complexes.[28,30] The predicted RNA–scaffold conformation was selected among the top-ranked solutions by virtue of the estimated binding affinity and the specific intermolecular contacts. Energy minimization was performed for the final complexes by using AMBER 9[31] with the corresponding force fields for nucleic acids and organic molecules.

Results and Discussion

Retrosynthetic analysis and synthesis

From a retrosynthetic perspective (Scheme 2), general structure **I** could be the outcome of an olefin-functionalization reac-



Scheme 2. Retrosynthetic analysis of general structure I. A) Olefin functionalization and final deprotections; B) Ring-closing metathesis retron; C) Allylation; D) Organometallic addition; E) Appropriate protecting strategy, degradation, oxidation.

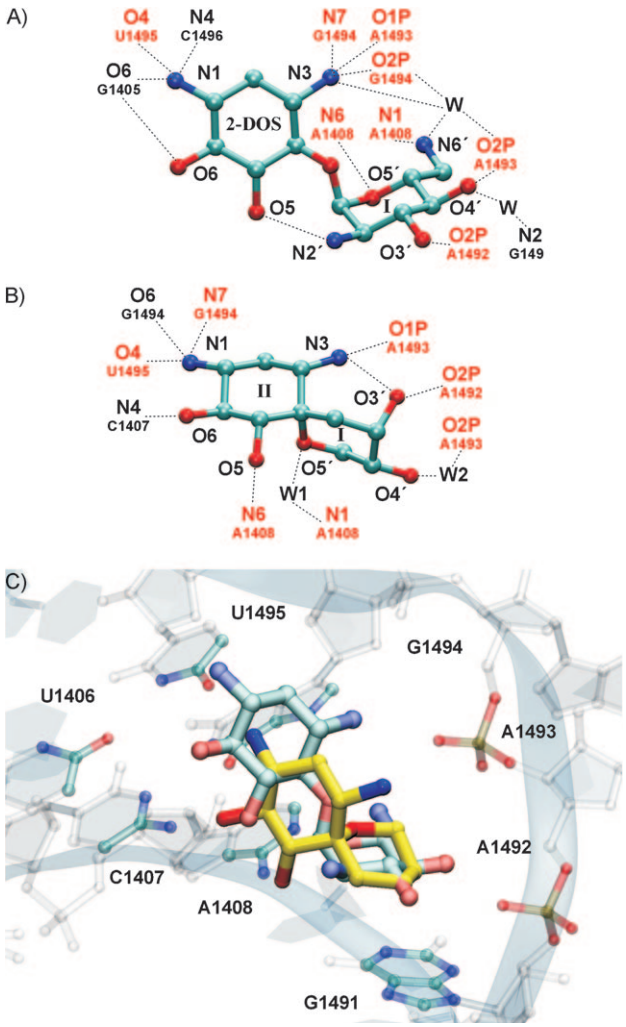
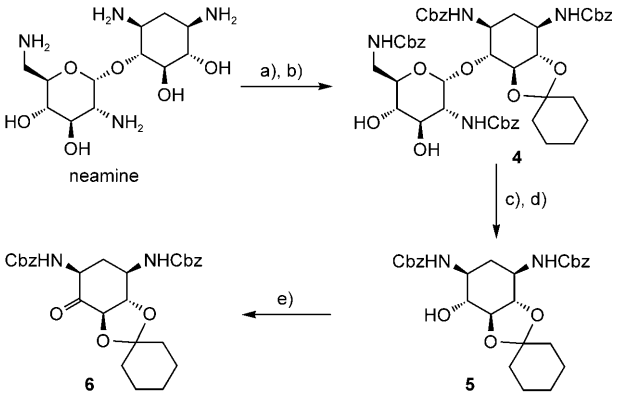


Figure 1. A) The contacts between neamine and the bacterial A-site RNA, extracted from the crystal structure with PDB ID 2ET8. Key conserved interactions among several aminoglycosides are marked in red. B) Simulated interactions between compound **2** and the bacterial A-site. W1 and W2 water molecules were modeled in proximity to conserved hydration sites of rRNA–aminoglycoside complexes. C) Predicted binding orientation of compound **I** (carbon atoms in yellow) superimposed with the crystallographic position of neamine (cyan carbons) into the bacterial A-site. Nitrogen atoms are colored blue, oxygen atoms are red, and phosphorus atoms are brown.

tion from intermediate **II**, which in turn could result from a ring-closing metathesis transformation of diene **III**. The specific diene could be formed through an allylation reaction of tertiary alcohol **IV**, the product of an organometallic addition to ketone **V**. Depending on the size of the organometallic reagent, vinyl, allyl, or homoallyl, the specific approach could provide access to a series of designed spiro-analogues with variations on the size of the newly formed ring (five, six, and seven). Finally, the corresponding ketone would be the outcome of a series of transformations, including appropriate protections of the functionalities present, degradation of neamine and oxidation of the resulting chiral 2-DOS derivative. Our approach towards the designed analogues was expected, and eventually proven to be chemically challenging due to the high density of polar functionalities present in their structures and the variable reactivity characteristics, which are exhibited from intramolecular interactions.

Synthesis of key intermediate ketone 6 (retron E)

The synthesis of the desired spiro ethers commenced with alcohol **5**, a common precursor to all of our analogues (Scheme 3). This adduct was readily available from the monoketal of tetra-benzyloxycarbonyl (Cbz)-protected neamine[23] **4**

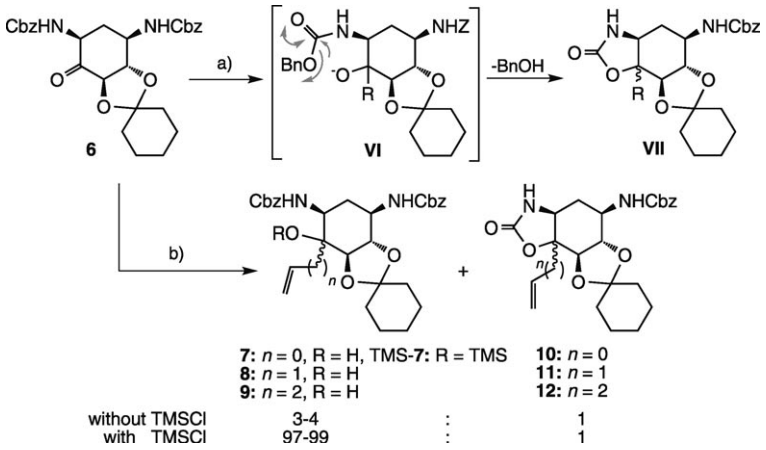


Scheme 3. Reagents and conditions: a) neamine (1.0 equiv), Cbz-Cl (7.5 equiv), $NaHCO_3$ (17.0 equiv), H_2O/dioxane (2:1 *v/v*), 12 h, 0 °C→25 °C; b) Dimethoxycyclohexane (7.5 equiv), *p*-TsOH (0.1 equiv), DMF (0.08 M), 5 h, 50 °C, 95% over two steps; c) $NaIO_4$ (6.0 equiv), MeOH (0.012 M), 0 °C→25 °C, 14 h; d) Et_3N (2.5 equiv), MeOH (0.015 M), 40 °C, 14 h, 94% over two steps; e) DMP (4.0 equiv), $NaHCO_3$ (6.0 equiv), CH_2Cl_2 (0.07 M), 50 °C, 1 h, 90%. DM [O] = Dess–Martin oxidation; CbzCl = benzyl chloroformate; *p*-TsOH = *p*-toluenesulfonic acid; DMF = dimethylformamide; DMP = Dess–Martin periodinane.

upon oxidative cleavage of its 1,2-diol and subsequent chromatographic separation (Scheme 3). Initial attempts to oxidize alcohol **5** to ketone **6**, under relatively mild conditions with oxalyl chloride/DMSO/Et_3N (Swern)[32] or I_2/KI[33] were not successful, and only the starting material could be recovered. A reliable solution proved to be the Dess–Martin periodinane,[34] which upon reflux with alcohol **5** furnished the desired ketone cleanly.

Organometallic additions to ketone 6 (retron D)

Based on our original design, ketone **6** would react with a Grignard reagent to afford the corresponding tertiary alcohol intermediate. Our initial attempts involved the addition of vinyl magnesium chloride to the electrophilic carbonyl and resulted in the isolation of the corresponding tertiary alcohol **7** along with oxazolidinone **10** (Scheme 4). The latter was formed presumably from the attack of the neighboring oxyanion to the carbonyl of the Cbz-group, with concomitant release of benzyl alcohol, which was in turn identified in the reaction products (**6**→**VI**→**VII**, Scheme 4). Similar results were obtained for the allylic and homoallylic organometallic reagents that form oxazolidinones **11** and **12**, respectively. Products **10**–**12** were also synthesized by conventional methods (NaH, DMF) in



Scheme 4. Reagents and conditions: a) organometallic bromide or chloride (3.2 equiv), THF (0.1 M), −78→25 °C, 4 h, 32% for oxazolidinone **10**, 35% for oxazolidinone **11**, 37% for oxazolidinone **12**; b) TMSCl (3.2 equiv), organometallic bromide or chloride (3.2 equiv), THF (0.1 M), −78→25 °C, 4 h, 36% for tertiary alcohol **7** (2.5:1 *dr*), 25% for tertiary TMS ether TMS-**7** (1:1.7), 68% for tertiary alcohol **8** (4:1 *dr*), 60% for tertiary alcohol **9** (7:1 *dr*). THF = tetrahydrofuran; TMS = trimethylsilyl.

high purity and yields for direct comparisons (Supporting Information).

The undesired outcome was circumvented through the inclusion of trimethylsilyl chloride (TMSCl) in the reaction mixture. The oxyanion was trapped in situ as the corresponding TMS-ether, which could be easily cleaved during the final workup, thus preventing the sequential formation of the oxazolidinone byproduct. Consequently, all three required alcohols **7**–**9** (vinyl, allyl, and homoallyl) were successfully synthesized (Scheme 4).

Regarding the stereoselectivity of the specific addition, it appears that the determining factor is the size of the organometallic reagent. Thus, alcohol **7** was isolated as a 2.5:1 mixture of isomers, alcohol **8** as a 4:1 mixture and alcohol **9** as a 7:1 mixture, as determined by HPLC analysis and ^{1}H NMR spectroscopy (Scheme 4). In all cases the major product was bearing the olefinic substituent in the equatorial position (*anti* to the adjacent NHR and OR′ functionalities, C4-*S*), as it was unequivocally established in the proceeding steps. Specifically regarding the vinylic addition, the corresponding equatorial (inversed stereoselectivity) TMS-ether TMS-**7** was also isolated in 25% yield, indicating different hydrolysis rates of the ethers; the axial TMS hydrolyzed faster than the equatorial TMS group. Thus, the actual selectivity of the isolated nucleophilic attack in this case was calculated to be 1.3:1.

Further trials to perform the observed in situ trapping of the tertiary oxyanion with allylbromide proved unsuccessful. Obviously in the specific case, the nature of the electrophile affects the organometallic addition directly, leading either to a complex reaction mixture through partial and uncontrolled removal of the protecting groups, or to the formation of the same oxazolidinone products **10**–**12**.

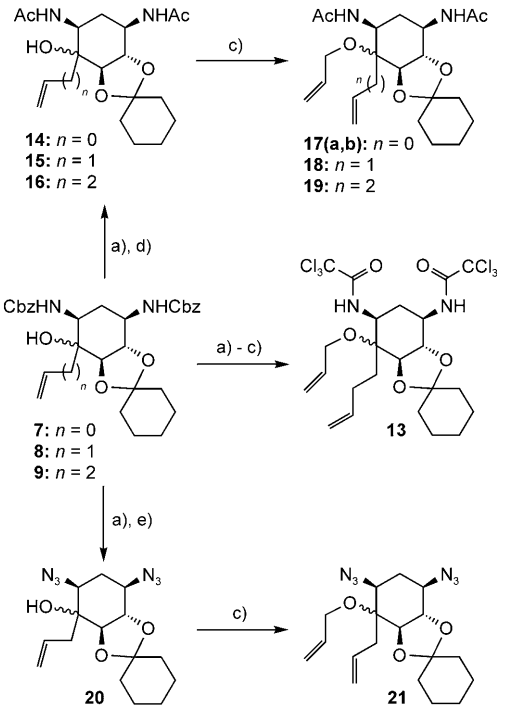
Alkylation of tertiary alcohols 7–9 (retron C)

Our attempts to etherify tertiary alcohols **7**–**9** with NaH resulted yet again in the formation of the oxazolidinone byproducts **10**–**12**, by the aforementioned putative mechanism (Scheme 4). Alternatively, when other basic conditions were employed (Ag_2O, iPr_2EtN, lithium bis(trimethylsilyl)amide (LiHMDS)), with tetrabutylammonium iodide (TBAI) as catalyst and allylbromide as the electrophilic reagent, only the unreacted alcohol was recovered. The trichloroacetimidate allylic moiety was also assayed under mildly acidic conditions, but still the desired etherification could not be affected.

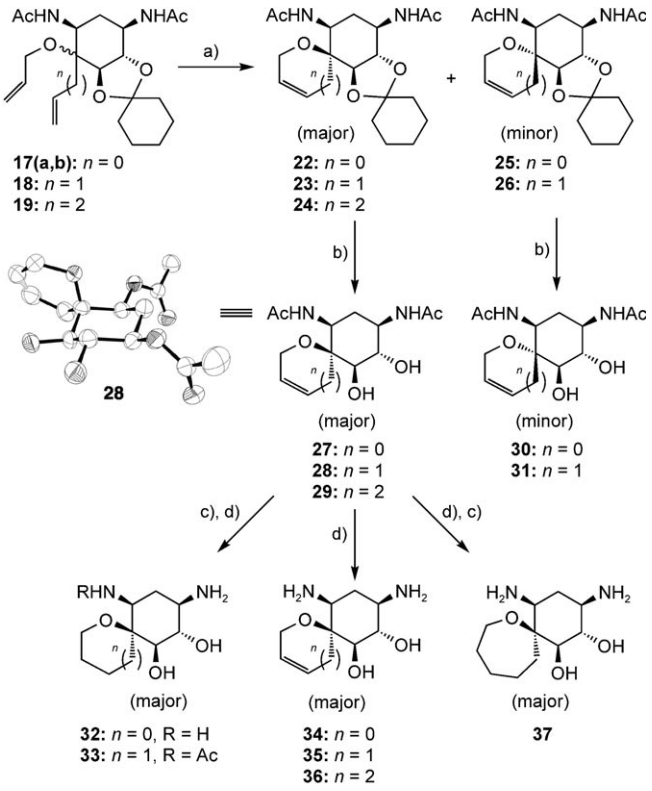
Careful examination of alternative routes, prompted us to remove the Cbz groups under strongly basic conditions because a hydrogenation could affect the existing double bonds. The specific transformation was successfully accomplished by the use of KOH, resulting in the synthesis of the desired diamines in high yields. Three different protecting strategies were selected at this stage for our synthesis, based on the ability of the protecting group to withstand the reaction conditions of the following steps, as well as our potential to remove them towards the end of the synthesis, without affecting the functionalities present in the final molecules. Initially, the diamine was successfully protected as the di-trichloroacetamide that further reacted with sodium hydride and allylic bromide[35] toward the desired allylic ether **13**. The more stable acetyl group was also selected for the protection of the diamines as the di-acetamides **14**–**16**. Its etherification proceeded smoothly to yield dienes **17**–**19** with high efficiency. Chromatographic separation of the two diastereomers was successfully realized only for diene **17**, leading to the characterization of **17a** (major isomer, vinyl group in equatorial position) and **17b** (minor isomer). In the course of our synthetic route we also experimented with the potential benefit of using an azide to mask the amines. Through a similar reaction sequence, diazide **20** became readily available and was further subjected to allylbromide addition toward ether **21** (Scheme 5).

Ring-closing metathesis (RCM) transformation for the synthesis of the spiro-skeleton (retron B)

Dienes **17**–**19** were subjected to a ring-closing metathesis with Grubbs' second-generation catalyst[36] to furnish the corresponding spiro ethers **22**–**26** as single isomers, after careful chromatographic separation (Scheme 6). The minor isomer of the seven-membered spiro-analogue was never observed, due to the very small quantities present. The di-trichloroacetamide diene **13** as well as the diazide analogue **21** were subjected to the same reaction conditions, but to our disappointment the desired products were formed only in trace amounts and were never isolated. Upon deprotection of the ketal functionality under acidic conditions diols **27**–**31** were isolated in high yields. Furthermore, diol **28** produced crystallographic data



Scheme 5. Reagents and conditions: a) KOH (aq), MeOH/H_2O (1:1 *v/v*), 100 °C, 14 h; b) $Cl_3CC(O)CCl_3$ (10 equiv), $CHCl_3$, 25 °C, 12 h; then $NaHCO_3$ (aq), MeOH/H_2O (1:1 *v/v*), 25 °C, 45 min, 37% for two steps; c) NaH (1.2 equiv), DMF (0.1 M), allylbromide (1.3 equiv), 0→25 °C, 10 h; d) Ac_2O (5.0 equiv), Et_3N (9.0 equiv), CH_2Cl_2, 25 °C, 2 h; then K_2CO_3 (2.0 equiv), MeOH, 25 °C, 3 h, 50–72% for two steps; e) TfN_3 (10 equiv), $CuSO_4$ (0.15 equiv), Et_3N (6.0 equiv), MeOH/H_2O (4:1), 25 °C, 20 h, 88%; Tf = triflic = trifluoromethylsulfonic.



Scheme 6. Reagents and conditions: a) RCM: Grubbs 2nd generation (0.1 equiv), CH_2Cl_2 (0.005 M), 25 °C, 18 h, 60–88% for the two isomers; b) AcOH/THF/H_2O (3:3:1 *v/v/v*), 40 °C, 18 h, 75–95%; c) H_2, Pd/C (cat.), MeOH, 25 °C, 18 h; d) KOH (70 equiv), H_2O (1 M), 120–150 °C, 14–48 h.

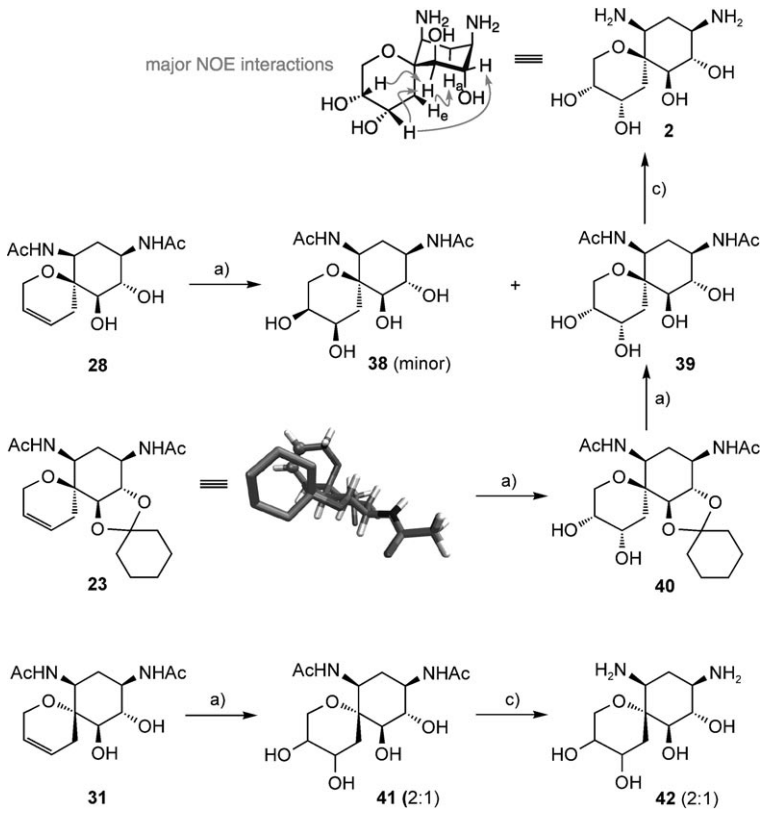
that confirmed the relative stereochemistry of this substrate (Scheme 6).

For the purpose of direct biological comparison, hydrogenation of the double bond in **27**–**28** followed by hydrolysis of the acetates furnished saturated spiro ethers **32** and **33** (Scheme 6). Cleavage of the N3 acetate in **33** could not be achieved even at high temperatures or extended reaction times. The analogous unsaturated compounds were also synthesized by basic treatment, as presented in Scheme 6. The order of the two final transformations could be inverted, as exemplified for 7-membered spiro ether **37** from diacetate **29**.

Dihydroxylation of olefins and final deprotections (retron A)

Dihydroxylation of olefin **28** under the standard Upjohn conditions[37] furnished a mixture of diastereomeric vicinal diols in 1:9 ratio (Scheme 7). Based on literature reports[38] regarding the selectivity of this reaction, we would expect it to proceed predominantly toward product **39** (addition of OsO_4 from the less-hindered site) because the stereochemically encumbered side is that facing the two hydroxyl groups of the 2-DOS-component. To test this hypothesis, the dihydroxylation was also assayed on the precursor acetal **23**. The optimal experimental conditions toward an enantiopure diol were found to be 0 °C for 3 h (98:2 *dr*). Further deprotection to the tetraol **39** and comparison of its ^1H NMR spectrum with material obtained by the aforementioned reverse synthetic protocol (ketal deprotection→dihydroxylation), confirmed the significant improvement in the stereoselection of the final product (Scheme 7). The final deprotection of the acetamide was accomplished with an aqueous solution of KOH and heating up to 130 °C overnight. Attempts to remove the acetates with controlled microwave heating (170 °C, 10 min) were unsuccessful (incomplete reactions and mixtures were obtained). Purification of the final product **2** by flash chromatography and repeated co-evaporations to dryness with a 0.1 M aqueous solution of HCl, furnished its di-hydrochloride salt that was delivered for immediate biological evaluation. The indicated stereochemistry of diamine **2** was assigned from the NMR spectroscopic data (NOESY, COSY). Strong NOE interactions were measured among characteristic hydrogen atoms of the bicyclic system (Scheme 7).

A similar synthetic pathway was also pursued for the more polar, minor spiro ether epimer **31**, which was dihydroxylated with catalytic amounts of osmium tetroxide to furnish tetraols **41** in 2:1 ratio (Scheme 7). Attempts to improve the diastereoselectivity in this case were not successful because, similarly to the previous rational, the reacting double bond is exposed to less steric hindrance than the epimeric spiro ether **28**. Thus, the stereocontrol elements that favor attack of the oxidizing agent from a certain direction are not in place, and a mixture of products is formed. The two tetraols **41** could be separated to the individual diastereomeric components by careful chro-



Scheme 7. Reagents and conditions: a) OsO_4 (0.1 equiv), NMO (3.0 equiv), acetone/H_2O (1:1 *v/v*), 0 °C or 25 °C, 3 h, 78–90%; b) AcOH/THF/H_2O (3:3:1 *v/v/v*), 40 °C, 18 h, 75–95%; c) KOH (70 equiv), H_2O (2 mL, 1 M), 130 °C, 15 h, 85–90%. NMO = *N*-methylmorpholine-*N*-oxide.

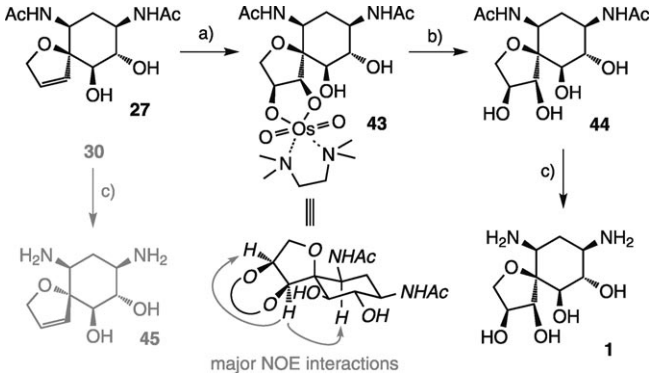
matographic purification. Furthermore, tetraols **41** were subjected to hydrolysis to furnish diamines **42**. Because biological evaluation of these isomers indicated significantly reduced potency to inhibit protein production in bacteria in comparison to **2**, as will be presented in the following section, assignment of their absolute stereochemistry was not pursued further.

For the more sterically hindered olefin **22** (Scheme 6) the standard Upjohn conditions proved ineffective, resulting in complete recovery of the starting material. The same result was also obtained for diol **27**. With stoichiometric amounts of osmium tetroxide though, olefin **27** was converted to the corresponding osmate ester **43**, which was isolated and further analyzed by two-dimensional NMR spectroscopy and NOE measurements (Scheme 8). These experiments indicated that the stereoselectivity of the hydroxylation in this case is opposite to that obtained for the six-membered analogue **39**. Apparently, the immediate proximity of the 5-OH to the double bond directs the oxidizing agent through a pre-coordination effect.[39] Hydrolysis of the ester with catalytic amounts of hydrochloric acid furnished tetraol **44**, which was further converted to diamine **1**. Noticeably, removal of the acetates in this case required higher temperatures and prolonged reaction times. Similarly, spiro-olefin **30** produced final diamine **45** (Scheme 8).

An analogous synthetic pathway was applied for the formation of the seven-membered spiro derivatives **3** and **50** (Scheme 9). In this case, dihydroxylation of olefin **24**, under standard conditions,[37] furnished a 2:3 mixture of diastereomeric vicinal diols. Attempts to improve the stereoselectivity of the reaction for the specific substrate, even by the use of AD-mix-α, were not successful. The differential topology of the double bond and the higher flexibility of the seven-membered ring, in comparison to its five- and six-membered counterparts, might be responsible for the observed outcome.

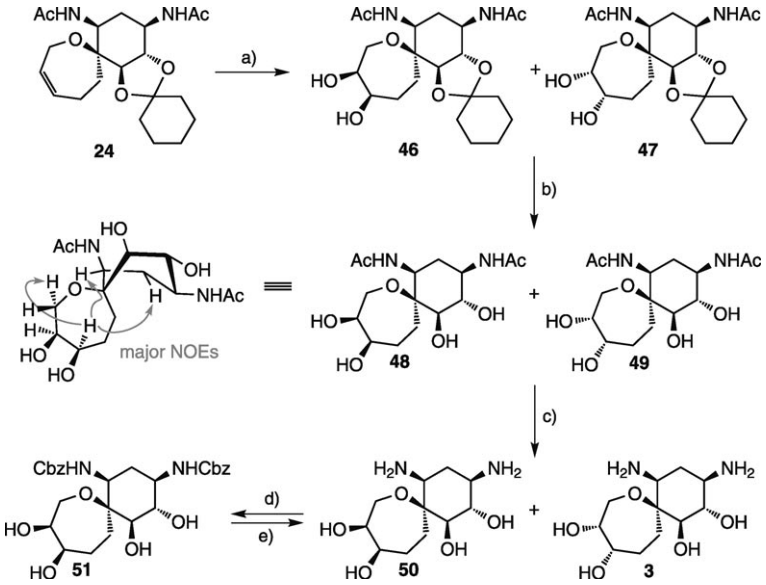
Removal of the ketal functionality in **46** and **47** under acidic conditions produced the corresponding tetraols **48** and **49** respectively. Two-dimensional NMR spectroscopy experiments at this stage allowed us to assign the absolute stereochemistry of the two compounds, as presented in Scheme 9. Final hydrolysis of the acetamides, followed by careful chromatographic separation, afforded the desired spiro ethers **50** (minor) and **3** (major).

Due to the highly polar nature of our final products in combination with the relatively harsh experimental conditions during the last steps of the synthesis, the inorganic salt, as well as the silica gel content of our samples represented a major consideration because they could directly affect the validity of the produced biological results. Thus, the final compounds were routinely purified with sephadex columns. Specifically, for compound **50** and only for the direct comparison of its biological potency regarding



Scheme 8. a) OsO_4 (1.2 equiv), TMEDA (1.2 equiv), CH_2Cl_2, −78 °C→25 °C, 3 h, 75%, b) MeOH, HCl (cat.), 25 °C, 15 min, quant., c) KOH (70 equiv), H_2O (2 mL, 1 M), 140 °C, 48 h, 20%.

its overall purity, a "chemical purification" method was applied. Thus, **50** was re-protected as the di-Cbz carbamate, then purified and deprotected by a catalytic hydrogenation reaction (Scheme 8). The potencies of the two samples, regarding their binding affinities for the RNA A-site construct were evaluated and the obtained values were identical. Additionally, quantification of the substances delivered for biological evaluation was performed by NMR spectroscopy. The method utilized is adequately described in the literature[40] and has been successfully employed by us in the past.[41] It utilizes as an internal



Scheme 9. Reagents and conditions: a) OsO_4 (0.1 equiv), NMO (3.0 equiv), acetone/H_2O (1:1 *v/v*), 0 °C or 25 °C, 3 h, 70%; b) TFA/THF/H_2O (3:3:1 *v/v/v*), 50 °C, 18 h, 75%; c) KOH (70 equiv), H_2O (2 mL, 1 M), 120 °C, 14 h, 70%; d) Cbz-Cl (4.0 equiv), $NaHCO_3$ (8.0 equiv), H_2O/dioxane (2:1 *v/v*), 14 h, 0 °C→25 °C; e) Pd/C, H_2, MeOH, 20 h.

standard 2,5-dimethylfuran (DMFu) to assess the amount of substance contained in the NMR spectroscopic solution by direct integration of the appropriate peaks. All our measurements indicated an excellent agreement between the sample's weight and its spectroscopic quantitation; this suggests the complete removal of all impurities during the selected purification process.

Biological evaluation of synthetic analogues

To evaluate the affinity of our synthetic analogues for the bacterial decoding-site, an RNA fluorescence assay was employed. A model RNA oligonucleotide was used in which the adenine at position 1492 has been replaced by a fluorescent probe, 2-aminopurine (2AP).[42] The probe's fluorescence is quenched when it is found stacked with other bases, but it increases upon compound binding and its exposure to solvent. We performed therefore titrations of compounds to the 2AP-labelled RNA double stranded construct, and increase in fluorescence intensity was monitored (Table 1, RNA specific assay). As a control, compounds were also titrated to 2AP-labeled single-stranded oligonucleotide with which no significant changes in fluorescence were observed. Representative plots are presented in Figure 2A–C.

The biological activity of the compounds, as functional bacterial-translation inhibitors, was evaluated in a coupled in vitro transcription-translation assay (IVT; Table 1, Bacterial IVT).[25] A gene encoding for firefly luciferase, as the reporter enzyme, was expressed in vitro by using bacterial S30 extract (Promega) in a mixture of nucleotide triphosphates, amino acids, and polymerase. The produced enzyme was quantified by subsequent addition of luciferin substrate, giving rise to a luminescence value relative to the amount of enzyme present. Compounds able to interfere with protein synthesis inhibit enzyme production, and therefore reduce luminescence (Figure 2D).

Aminoglycosides are also known to interfere with eukaryotic protein synthesis. Some of them have the ability to induce mammalian read-through stop-codon mutations and generate fully functional proteins in several genetic disorders.[43] Therefore, we investigated the performance of these compounds towards eukaryotic protein translation inhibition in vitro. Similarly to the bacterial IVT, luciferase is expressed from a plasmid containing the encoding gene under the command of T7 promoter, in a mixture that combines RNA polymerase, reticulocyte lysate solution and nucleotides. Compounds that interfere with protein synthesis cause a decrease in the observed luminescence (Table 1, Eukaryotic IVT).

As indicated in Table 1 and Figure 2, many of our synthetic analogues exhibited satisfactory binding affinities for the bacterial decoding center, with the 6,6- and 6,5- scaffolds producing the best results. Interestingly, a significant variability was observed regarding the efficacy of the fluorescent measurement. For compounds **2**, **42**, **50**, and **3** that exclusively generated an effective fluorescent signal upon binding, these affinities are also supported by their potencies as inhibitors of bacterial protein production. Especially for compound **2** (EC_{50} = 6.5 μM), the observed values are of great importance because they are directly comparable to neamine, despite the fact that the designed analogue possesses half the electrostatic load present in the natural product (EC_{50} = 9.5 μM), with only two amino groups

Table 1. Biological activities for natural aminoglycosides and spiro derivatives.

Compound	RNA EC_{50} [μM][b]	$B_{IVT}IC_{50}$ [μM][a]	$E_{IVT}IC_{50}$ [μM][c]
neomycin B	0.007	0.032	252
neamine	9.5	5	120
2	6.5	11.3	no activity
42	9.3	174	no activity
35	3.2[d]	no activity	no activity
33	18[d]	no activity	no activity
50	74	118	130
3	141	168	160
36	8.4[d]	no activity	no activity
37	no binding	no activity	no activity
1	1.5[d]	187[d]	no activity
34	1.7[d]	no activity	no activity
45	2.4[d]	no activity	no activity
32	0.358[d]	no activity	no activity

[a] RNA EC_{50} values were determined by decoding-site RNA fluorescence assay and calculated as the average of three replicate experiments for each compound (±10%); [b] $B_{IVT}IC_{50}$ values were determined by coupled transcription–translation assay by using *E. coli* extract, calculated as the average of three replicate experiments for each compound (±15%); [c] $E_{IVT}IC_{50}$ values were determined by TNT quick-coupled transcription–translation assay, as the average of three replicate experiments per compound (±15%); [d] Low efficacy in effect measured.

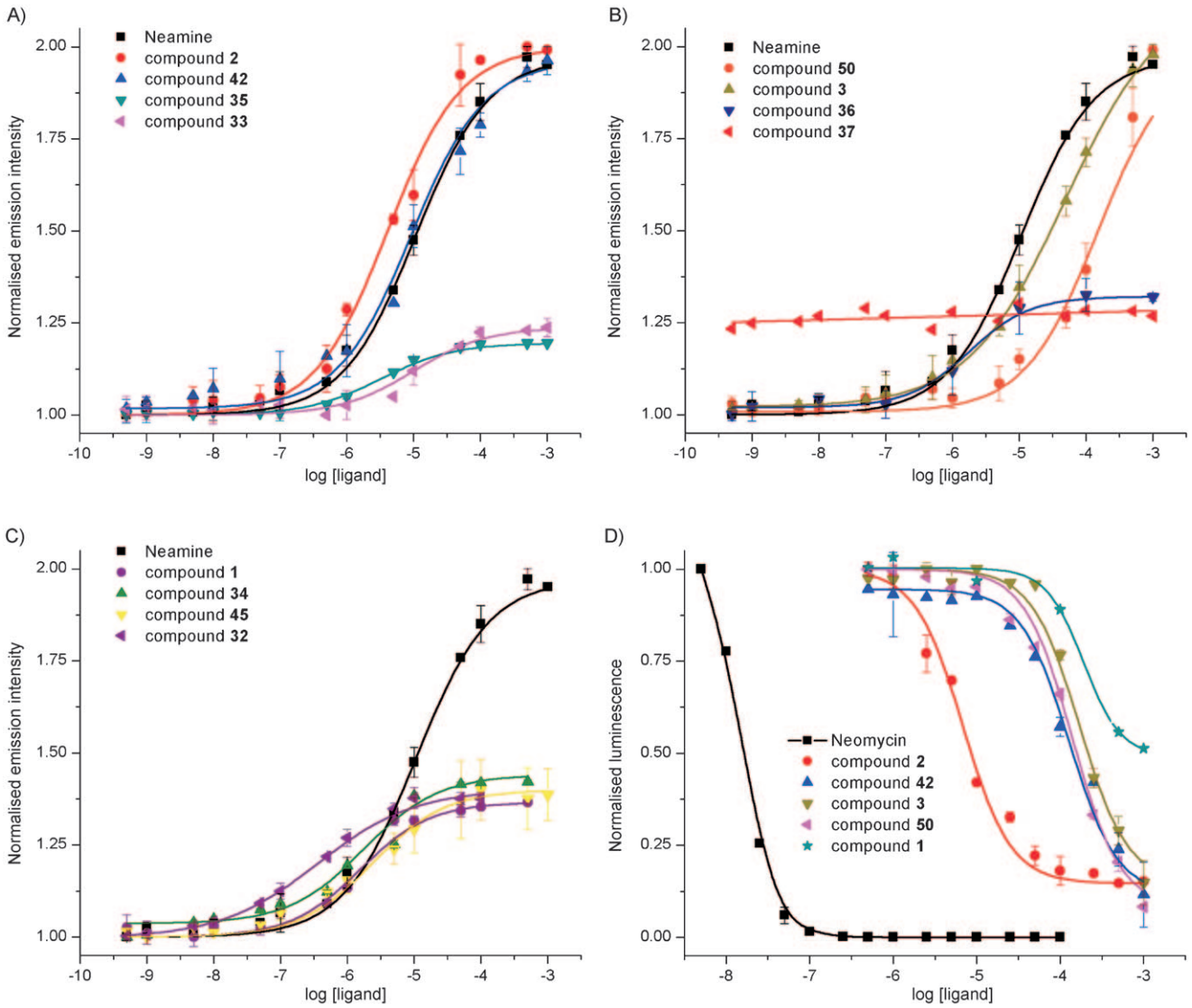


Figure 2. Normalized fluorescence signal at 370 nm during titration of neamine and A) 6,6-spiro compounds **2**, **42**, **35**, and **33**, B) 7,6-spiro ligands **50**, **3**, **36**, **37**, and C) 5,6-spiro analogues **1**, **34**, **45**, **32** to RNA construct probed with 2AP label at residue 1492. D) Normalized Luminescence Units, corresponding to bacterial in vitro transcription-translation, versus log(c) plots of neomycin and synthetic compounds **2**, **42**, **3**, **50**, and **1** by fitting to a variable slope dose–response equation.

embedded in its structure. The ability of 7-membered counterparts **50** and **3** to maintain similar values between bacterial and eukaryotic protein synthesis inhibition is also noticeable. Regarding compounds **1**, **32**–**35**, and **45**, the obtained EC_{50} values were calculated based on lower-efficacy fluorescent measurements, as presented in Figure 2. We speculate that this observation could be consistent with a smaller conformational change of the RNA target, which overall cannot sufficiently alter the mechanism of protein production.

The similarities observed in the values of most analogues in conjugation with our molecular modeling analysis lead to a conserved, yet not fully optimized, binding orientation, in which important interactions are preserved. For example, in the simulated complex of **2** with the A-site (Figure 1 B), N1 of **2** interacts with U1495 and G1494, whereas N3 is involved in electrostatic interactions with the phosphate group of A1493. The hydroxyls O5 and O6 were found to exhibit major contacts with C1407 and A1408, whereas O3′ forms direct contacts with the phosphate of A1492. In the most favorable orientation, the ether moiety of **2** is predicted to be stacked onto G1491 in the same manner as ring I of all natural aminoglycosides. The conserved interaction with A1408 is mediated by O5 of **2** and probably a water molecule (W1 in Figure 1 B), which was modeled at the position occupied by N6′ of neamine's ring I (Figure 1 A). A second water molecule (W2 in Figure 1 B) substituting O4′ of neamine, could potentially mediate a hydrogen bond with O2P of A1493.

Conclusions

By diverging from previous research efforts, which focused on the direct modification of natural aminoglycosides and simpli-

fied analogues, we successfully constructed compounds that are able to mimic the bonding interactions of the natural products for the ribosomal decoding center. Specifically, rigid spirocyclic scaffolds have been designed and the development of an optimized synthetic route for their production is described. All final products were tested for their ability to bind to the bacterial decoding center, as well as for their potential to inhibit protein production in vitro. Our results formulate a preliminary SAR study for the identification of important interactions and support the development of analogues with improved antibiotic profiles. Six-membered spirocyclic scaffold **2** represents the champion of the present effort and is able to maintain the desired activity with half the number of the notorious amino moieties present in neamine. Also, direct comparison between the 6,6- and 7,6-analogues introduces the possibility of controlling the selectivity of action between bacteria and eukaryotic organisms through fine tuning of structural elements. Additional examples, co-crystallographic studies and biological evaluations are currently underway in our laboratory.

Experimental Section

Unless otherwise noted, solvents and reagents for organic synthesis were obtained from commercial suppliers and were used without further purification. Tetrahydrofuran (THF), toluene and diethyl ether were distilled from sodium benzophenone, and dichloromethane (CH_2Cl_2) was distilled from calcium hydride. All reactions were carried out under a dry argon atmosphere with anhydrous, freshly distilled solvents under anhydrous conditions unless otherwise noted. All reactions were magnetically stirred with Teflon stir bars, and temperatures were measured externally. Reactions requiring anhydrous conditions were carried out in oven dried (120 °C, 24 h) or flame dried (vacuum <0.5 Torr) glassware. Yields refer to chromatographically and spectroscopically (^{1}H NMR) homogeneous materials. All reactions were monitored by thin layer chromatography (TLC) carried out on 0.25 mm E. Merck silica gel plates (60F-254) by using UV light as visualizing agent and 7% ethanolic phosphomolybdic acid or *p*-anisaldehyde solution and heat as developing agents. E. Merck silica gel (60, particle size 0.040–0.063 mm) was used for flash column chromatography. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AM-250 or a Bruker Avance DRX-500 instrument as noted individually. Unless stated otherwise, NMR spectroscopy experiments were performed at 25 °C. Chemical shifts are measured in parts per million (δ) relative to the deuterated solvent used in the experiment.

1,3,2′,6′-Tetra-Cbz-5,6-O-cyclohexylidine neamine 4: Tetra-Cbz-neamine (22.4 g, 26.1 mmol) and *p*-toluenesulfonic acid (*p*-TsOH; 554 mg, 2.91 mmol) were dissolved in dry DMF (150 mL). While heating the solution at 50 °C, 1,1-cyclohexanone dimethyl ketal (29.8 mL, 195 mmol) was added. The solution was stirred at 50 °C for 5 h. At this point the starting material was consumed and a mixture of mono- and diketal was formed ($R_f=0.5$ and 0.85 respectively, 5% MeOH/CH_2Cl_2). MeOH (10 mL) was added, and the mixture was stirred for 1 h, at 50 °C while monitored by TLC. Et_3N (9.0 mL) was finally added, and the reaction mixture was cooled to RT. H_2O (500 mL) was added slowly to the stirred solution leading to the formation of a precipitate. The solids were filtered off and washed successively with H_2O (250 mL) and ice-cold Et_2O (2× 250 mL). The white solid was dried under reduced pressure and finally overnight on high vacuum to obtain **4** (23.2 g, 95% yield); $R_f=0.51$ in 5% MeOH/CH_2Cl_2; ^{1}H NMR (500 MHz, [D$_6$]DMSO): $\delta=$ 7.48 (d, $J=8.0$ Hz, 1H), 7.41–7.24 (m, 20H), 7.23 (d, $J=7.7$ Hz, 1H), 7.01 (brs, 1H), 6.56 (brs, 1H), 5.13–5.07 (m, 2H), 5.06–4.97 (m, 6H), 4.97–4.90 (m, 2H), 4.89–4.85 (brs, 1H) 3.74–3.62 (m, 4H), 3.58 (ddd, $J=8.8$, 9.0, 9.0 Hz, 1H), 3.49–3.34 (m, 4H), 3.28–3.19 (m, 1H), 3.13 (brs, 1H), 1.93–1.82 (m, 1H), 1.59–1.28 (m, 10H), 1.28–1.15 ppm (m, 1H); ^{13}C NMR (125 MHz, [D$_6$]DMSO): $\delta=$156.6, 156.0, 155.6, 155.4, 137.1, 137.0, 136.9 (2C), 128.3 (4C), 128.2, 127.8, 127.7 (2C), 127.6 (3C), 127.5, 110.8, 96,1, 80.2, 77.8, 76.1, 71.4, 70.4, 70.0, 65.4, 65.3 (3C), 55.5, 50.8, 48.3, 41.9, 36.1, 35.6, 24.5, 23.3, 23.1 ppm; HRMS-ESI: *m/z*: calcd for $C_{50}H_{58}N_4NaO_{14}$: 961.3847 [*M*+Na]$^+$, found: 961.3840.

Synthesis of benzyl (3a*S*,4*R*,5*S*,7*R*,7a*S*)-4-hydroxyhexahydrospiro[benzo[d][1,3]dioxole-2,1′-cyclohexane]-5,7-diyldicarbamate (5): The monoketal **4** (15.0 g, 16.0 mmol) was suspended in MeOH (800 mL) and cooled to 0 °C. $NaIO_4$ (20.5 g, 95.9 mmol) was added, the reaction flask was wrapped with aluminum foil, and the mixture was stirred vigorously overnight. The solids were filtered off, and residual solvent was removed at high vacuum overnight. Quick purification by column chromatography ($CH_2Cl_2\rightarrow$10% MeOH/CH_2Cl_2) afforded the corresponding dialdehyde as a white amorphous solid (15.0 g, quant.; $R_f=0.7$ in 5% MeOH/CH_2Cl_2), which was used directly in the next step.

The corresponding dialdehyde (0.44 g, 0.47 mmol) was dissolved in MeOH (33 mL), Et_3N (0.16 mL, 1.2 mmol) was added, and the reaction mixture was stirred vigorously at 40 °C for 13 h. The mixture was concentrated under reduced pressure, and the residue was purified by column chromatography (20→60% EtOAc/hexane) to afford product **5** as a white amorphous solid (226 mg, 94% yield). If necessary, an additional recrystallization by using EtOAc/*n*-hexane could be performed. $R_f=0.69$ in 35% EtOAc/CH_2Cl_2; $[\alpha]_D^{22}=-5.6°$ ($c=0.6$, MeOH); ^{1}H NMR (500 MHz, CD_3OD): $\delta=$7.33–7.27 (m, 10H), 5.06 (brs, 4H), 3.78 (brs, 1H), 3.57–3.53 (m, 2H), 3.44–3.42 (m, 2H), 2.15 (ddd, $J=4.4$, 4.4, 13.3 Hz, 1H), 1.65–1.60 (m, 8H), 1.40–1.28 ppm (m, 3H); ^{13}C NMR (125 MHz, CD_3OD): $\delta=$158.6, 158.2, 138.2 (2C), 129.4 (2C), 128.9 (2C), 128.8 (2C), 128.8 (2C), 113.0, 82.0, 79.7, 73.9, 67.5 (2C), 54.9, 50.4, 37.5, 37.3, 37.1, 26.1, 24.7 ppm (2C) ; HRMS-ESI: *m/z*: calcd for $C_{28}H_{34}N_2NaO_7$: 533.2264 [*M*+Na]$^+$, found: 533.2257.

Dess–Martin oxidation for the synthesis of benzyl (3a*R*,5*S*,7*R*,7a*S*)-4-oxohexahydrospiro[benzo[d][1,3]dioxole-2,1′-cyclohexane]-5,7-diyldicarbamate (6): The precursor alcohol **5** (1.25 g, 2.50 mmol) was dissolved in CH_2Cl_2 (37.5 mL) and $NaHCO_3$ (1.26 g, 15.0 mmol) was added. The suspension was allowed to stir for 10 min, the Dess–Martin periodinane (4.16 g, 10.0 mmol) was added and the mixture warmed to 50 °C for 1 h. A sat. aq solution of $Na_2S_2O_3$ (18 mL) was added, and the mixture was vigorously stirred until it became transparent. It was then extracted with $NaHCO_3$ (3×15 mL), dried ($MgSO_4$), and concentrated in vacuo. The solid crude product was purified by flash column chromatography on silica gel ($CH_2Cl_2\rightarrow$3% acetone/CH_2Cl_2) to afford ketone **6** as a white amorphous solid. (1.13 g, 90% yield); $R_f=0.9$ in 5% acetone/CH_2Cl_2; ^{1}H NMR (500 MHz, $CDCl_3$): $\delta=$7.41–7.27 (m, 10H), 4.56–4.50 (m, 1H), 5.15–5.06 (m, 4H), 4.93–4.85 (m, 1H), 4.51–4.42 (m, 1H), 4.32 (d, $J=10.2$ Hz, 1H), 4.25–4.16 (m, 1H), 3.67–3.55 (m, 1H), 3.09–2.27 (m, 1H) 1.76–1.44 (m, 9H), 1.43–1.28 ppm (m, 3H); ^{13}C NMR (125 MHz, [D$_6$]DMSO): $\delta=$198.4, 155.8, 136.2, 133.2, 131.3, 129.3, 128.6, 128.3, 114.1, 80.2, 67.2, 55.1, 49.4, 37.3, 36.4, 35.7, 24.8, 23.5 ppm; IR (neat): $\tilde{\nu}=$3032, 2938, 1704, 1526, 1250, 1058 cm^{-1}; HRMS-ESI: *m/z*: calcd for $C_{28}H_{32}N_2NaO_7$: 531.2107 [*M*+Na]$^+$, found: 531.2103.

Vinyl-Grignard addition to ketone 6. Synthesis of benzyl (3a*R*,5*S*,7*R*,7a*S*)-4-hydroxy-4-vinylhexahydrospiro[benzo[d][1,3]dioxole-2,1′-cyclohexane]-5,7-diyldicarbamate (7): At −78 °C, ketone **6** (587 mg, 1.15 mmol) was dissolved in anhyd THF (8.7 mL) and TMSCl (0.45 mL, 3.50 mmol) was added. A solution of vinylmagnesium chloride was added dropwise (1.6 M in THF, 2.2 mL, 3.5 mmol) and the stirred solution was allowed to warm to RT over 4 h. Then a sat. aq solution of NH_4Cl (4.0 mL) was added, and the mixture was extracted with EtOAc (3×8.0 mL). The organic solution was dried ($MgSO_4$), concentrated in vacuo, and purified by flash chromatography on silica gel (CH_2Cl_2→5% acetone/CH_2Cl_2) to obtain the corresponding allylic alcohols **7** (225 mg, 36% yield, 2.5:1 *dr*; R_f=0.32 in 5% acetone/CH_2Cl_2) as a white solid and the TMS ethers **7a** (154 mg, 25% yield, 1:1.7 *dr*; R_f=0.68 in 5% acetone/CH_2Cl_2) as colorless oils; **7:** ^{1}H NMR (500 MHz, $CDCl_3$): δ=7.30 (m, 10H), 5.86 (dd, J=11.0, 17.1 Hz, 1H; major), 5.69 (d, J=16.3 Hz, 1H; minor), 5.48 (J=10.6 Hz, 1H; minor), 5.39 (d, J=17.3 Hz, 1H; major), 5.24 (J=11.0 Hz, 1H; major) 5.06–5.03 (m, 4H+1H minor), 3.82 (m, 2H), 3.65 (m, 1H), 3.47 (m, 1H), 2.73 (br s, 1H), 2.32 (t, J=6.5 Hz, 1H), 1.84 (m, 1H), 1.65–1.35 ppm (m, 10H); ^{13}C NMR (125 MHz, $CDCl_3$): δ=156.9, 156.8, 156.0, 155.8, 139.7, 138.3, 136.3, 136.2, 136.1, 136.0, 129.0, 128.4, 128.0, 121.1, 116.4, 112.3, 111.7, 83.1, 81.3, 80.1, 76.3, 75.3, 74.8, 67.2, 67.1, 66.8, 66.6, 55.8, 52.5, 50.0, 49.4, 36.4, 36.2, 36.1, 35.9, 35.0, 29.6, 26.9, 24.9, 23.6, 23.5 ppm; IR (neat): $\tilde{\nu}$=3339, 2945, 1682, 1507, 1454, 1342, 1253, 1084, 1037, 697 cm^{-1}; HRMS-ESI: *m/z*: calcd for $C_{30}H_{37}N_2O_7$: 537.2595 $[M+H]^+$, found: 537.2593; **7a:** ^{1}H NMR (500 MHz, $CDCl_3$): δ=7.34 (m, 10H), 5.94 (dd, J=10.9, 17.6 Hz, 1H; minor), 5.85 (m, 1H; major), 5.58 (d, J=17.6 Hz, 1H; major), 5.51 (d, J=17.5 Hz, 1H; minor), 5.46 (d, J=10.7 Hz, 1H; major), 5.25 (d, J=11.2 Hz, 1H; minor), 5.14–5.05 (m, 4H), 4.87 (d, J=10.0, Hz, 1H; minor), 4.77 (m, 1H; major), 3.81–3.52 (m, 4H), 2.40 (m, 1H; major), 2.34 (t, J=6.8 Hz, 1H; minor), 1.65–1.20 (m, 11H), 0.17–0.15 ppm (m, 9H); ^{13}C NMR (125 MHz, $CDCl_3$): δ=156.1, 155.7, 137.3, 136.4, 132.9, 128.5, 128.1, 128.0, 121.0, 117.8, 111.8, 111.5, 82.8, 80.7, 78.6, 78.3, 75.8, 75.6, 67.3, 66.7, 66.6, 56.6, 54.6, 49.9, 48.3, 36.4, 36.3, 35.9, 35.0, 25.0, 23.8, 23.8, 23.6, 23.4, 2.6, 1.9 ppm; IR (neat): $\tilde{\nu}$=3327, 2937, 1700, 1516, 1453, 1251, 1046, 844, 738, 697 cm^{-1}; HRMS-ESI: *m/z*: calcd for $C_{33}H_{44}N_2O_7Si$: 608.2912 $[M+H]^+$, found: 608.2913.

Allyl-Grignard addition to ketone 6. Synthesis of benzyl (3a*R*,5*S*,7*R*,7a*S*)-4-allyl-4-hydroxyhexahydrospiro[benzo[d][1,3]dioxole-2,1′-cyclohexane]-5,7-diyldicarbamate (8): Similarly to **7** and with allylmagnesium bromide as the nucleophile, homoallylic alcohols **8** were isolated as a white solid (281 mg, 68% yield, 4:1 *dr*); R_f=0.24 in 40% EtOAc/hexanes; ^{1}H NMR (500 MHz, $CDCl_3$): δ=7.55–7.37 (m, 10H), 6.05 (m, 1H), 5.44–5.11 (m, 6H), 3.98–3.78 (m, 4H), 3.49 (m, 1H), 2.65 (br s, 1H), 2.54–2.41 (m, 3H), 1.85–1.44 ppm (m, 11H); ^{13}C NMR (125 MHz, $CDCl_3$): δ=155.8, 136.4, 132.3, 128.5, 128.4, 128.2, 120.1, 111.5, 80.6, 77.4, 76.9, 73.3, 67.0, 66.8, 58.8, 51.1, 49.6, 40.7, 36.4, 35.2, 25.0, 23.8, 23.7 ppm; IR (neat): $\tilde{\nu}$=3284, 3076, 2933, 2855, 1639, 1545, 1448, 1371, 1040, 920 cm^{-1}; HRMS-ESI: *m/z*: calcd for $C_{31}H_{38}N_2NaO_7$: 573.2577 $[M+Na]^+$, found: 573.2571.

Homoallyl-Grignard addition to ketone 6. Synthesis of benzyl (3a*R*,5*S*,7*R*,7a*S*)-4-(but-3-enyl)-4-hydroxyhexahydrospiro[benzo-[d][1,3]dioxole-2,1′-cyclohexane]-5,7-diyldicarbamate (9): Similarly to **7** and with 3-butenylmagnesium bromide as the nucleophile, homoallylic alcohols **9** were isolated (7:1 mixture of diastereoisomers, 0.24 g, 60% yield) as a white foam; R_f=0.24 in 40% EtOAc/hexanes; ^{1}H NMR (500 MHz, $CDCl_3$): δ=7.49–7.39 (m, 20H), 5.97–5.82 (m, 1H; major), 5.81–5.78 (m, 1H; minor), 5.31 (d, J=9.5 Hz, 2H), 5.26–5.14 (m, 8H), 5.09 (d, J=10.0 Hz, 2H), 3.89 (br s, 3H; major), 3.76 (br s, 3H; minor), 3.49 (d, J=7.0 Hz, 1H; major), 3.42 (d, J=6.5 Hz, 1H; minor), 2.47–2.32 (m, 6H), 2.31–2.19 (m, 2H), 2.00–1.79 (m, 4H), 1.78–1.63 (m, 20H), 1.60–1.52 ppm (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$): δ=156.3, 156.2, 138.8, 136.8, 129.0, 128.6, 128.5, 115.5, 115.2, 112.0, 81.2, 80.6, 76.0, 74.4, 67.3, 67.2, 52.5, 52.1, 49.9, 36.8, 36.7, 36.1, 35.9, 35.6, 28.7, 25.4, 25.2, 24.1, 24.0, 23.9, 23.8 ppm; HRMS-ESI: *m/z*: calcd for $C_{32}H_{40}N_2NaO_7$: 587.2733 $[M+Na]^+$; found: 587.2724.

Synthesis of *N*,*N*′-((3a*R*,5*S*,7*R*,7a*S*)-4-allyl-4-(allyloxy)hexahydrospiro[benzo[d][1,3]dioxole-2,1′-cyclohexane]-5,7-diyl)bis(2,2,2-trichloroacetamide) (13): Alcohol **9** (0.12 g, 0.21 mmol) and KOH (0.50 g, 8.9 mmol) were dissolved in H_2O (1.0 mL) and MeOH (1.0 mL) in a round-bottomed flask. The solution was warmed at 100 °C for 14 h and subsequently concentrated in vacuo to dryness. The solid residue was purified by flash chromatography on silica gel (MeOH→10% NH_4OH/MeOH) to furnish the corresponding epimeric (at the quaternary center, 7:1 *dr*) diamines (52 mg, 85%).

At 25 °C, under an argon atmosphere, the diamines (28 mg, 0.094 mmol) were dissolved in dry $CHCl_3$ (1 mL) containing hexachloroacetone (0.15 mL, 0.99 mmol) and allowed to stir for 12 h. The solvents were removed in vacuo and a solution of $NaHCO_3$ (20 mg, 0.24 mmol) in MeOH/H_2O (1:1, *v/v*) was added and allowed to vigorously stir for 45 min. The solvents were removed in vacuo and the mixture was purified by flash chromatography on silica gel (CH_2Cl_2 to 10% MeOH/CH_2Cl_2) to obtain the corresponding trichloroacetamides (20 mg, 37% yield; R_f=0.46 in 10% MeOH/CH_2Cl_2); $[\alpha]_D^{22}=-69.8°$ (c=0.8, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ=6.9 (m, 1H), 5.98 (m, 1H), 5.21 (d, J=17 Hz, 1H), 5.10 (d, J=10 Hz, 1H), 4.12–4.08 (m, 2H), 3.52 (m, 1H), 3.14 (m, 1H), 2.96–2.72 (br s, 1H), 2.47 (m, 1H), 2.45 (m, 2H), 2.04 (m, 1H), 1.82 (m, 1H), 1.81–1.65 (m, 10H), 1.52 ppm (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$): δ=161.8, 138.3, 115.0, 112.0, 92.3, 80.9, 74.6, 73.0, 51.7, 50.6, 36.3, 36.2, 35.2, 34.9, 28.3, 25.0, 23.7, 22.7 ppm.

At 0 °C, under an argon atmosphere, the corresponding alcohol (20 mg, 0.034 mmol) was dissolved in dry DMF (1.0 mL) and NaH (1.5 mg, 0.037 mmol, 60% *w/w*) was slowly added. The mixture was allowed to stir at this temperature for 30 min, then allyl bromide (5.0 μL, 0.040 mmol) was added, and the solution was slow warmed to 25 °C over 10 h. A sat. aq solution of NH_4Cl (2 mL) was added, and the mixture was extracted with EtOAc (2×5 mL). The organic solution was washed with H_2O (1 mL) and brine (1 mL), dried ($MgSO_4$) and concentrated in vacuo. The mixture was purified by flash chromatography on silica gel (CH_2Cl_2→10% MeOH/CH_2Cl_2) to obtain the corresponding ether **13** (9.0 mg, 43% yield); R_f=0.67 in 10% MeOH/CH_2Cl_2; ^{1}H NMR (500 MHz, $CDCl_3$): δ=5.87 (m, 2H), 5.19–4.95 (m, 5H), 4.80 (m, 1H), 4.11–4.03 (m, 1H), 3.32 (m, 1H), 3.64 (m, 1H), 3.39 (m, 1H), 2.51–2.11 (m, 5H), 1.91–1.59 ppm (m, 11H); ^{13}C NMR (125 MHz, $CDCl_3$): δ=162.5, 155.3, 138.2, 133.7, 116.4, 114.9, 81.8, 71.2, 56.7, 50.9, 46.9, 39.9, 36.8, 36.4, 35.8, 27.4, 26.2, 24.9, 23.7, 23.5 ppm.

Synthesis of *N*,*N*′-((3a*R*,5*S*,7*R*,7a*S*)-4-hydroxy-4-vinylhexahydrospiro[benzo[d][1,3]dioxole-2,1′-cyclohexane]-5,7-diyl)diacetamide (14): The Cbz-protected amine **7** (0.22 g, 0.41 mmol) was dissolved in H_2O/MeOH (4.0 mL, 1:1 *v/v*) and KOH (0.92 g, 16 mmol) was added. The solution was heated to 100 °C for 14 h, and the solvent was removed in vacuo. The crude product was purified by flash column chromatography on silica gel (MeOH→10% NH_4OH/MeOH) to furnish the free amines (61 mg, 55% yield). The amines (85 mg, 0.32 mmol) were dissolved in CH_2Cl_2 (1.0 mL) and cooled to 0 °C. Et_3N (0.40 mL, 2.8 mmol) and Ac_2O (0.15 mL, 1.6 mmol)

were added. The solution was stirred at RT for 4 h, and the solvent was removed in vacuo. The crude product was purified by flash column chromatography on silica gel ($CH_2Cl_2 \rightarrow 7\%$ MeOH/CH_2Cl_2) to furnish the acetates **14** (88 mg, 79% yield; $R_f = 0.38$ in 10% MeOH/CH_2Cl_2) as a white solid. ^{1}H NMR (500 MHz, CD_3OD): $\delta =$ 6.00 (dd, $J = 11.0$, 17.0 Hz, 1H; minor), 5.84 (dd, $J = 10.8$, 17.3 Hz, 1H; major), 5.62 (d, $J = 17.0$ Hz, 1H; minor), 5.48 (d, $J = 11.0$ Hz, 1H; minor), 5.41 (d, $J = 17.5$ Hz, 1H; major), 5.18 (d, $J = 11.0$ Hz, 1H; major), 4.10–3.93 (m, 3H; major+2H; minor), 3.70–3.63 (m, 2H; minor), 3.54 ppm (d, $J = 9.0$ Hz, 1H; major); ^{13}C NMR (125 MHz, CD_3OD): $\delta = 172.4$, 171.4, 171.4, 171.1, 138.9, 132.5, 119.3, 114.6, 111.3, 110.8, 83.3, 81.7, 76.3, 76.2, 75.1, 74.3, 54.4, 51.0, 47.7, 46.5, 36.1, 35.9, 35.9, 35.7, 34.2, 33.3, 24.7, 24.6, 23.3, 23.1, 21.3, 21.2, 21.1 ppm; IR (neat): $\tilde{\nu} = 3283$, 2937, 1653, 1554, 1448, 1281, 1118, 920 cm^{-1}; HRMS-ESI: *m/z*: calcd for $C_{18}H_{29}N_2O_5$: 353.2071 $[M+H]^+$, found: 353.2070.

Synthesis of *N,N′*-((3a*R*,5*S*,7*R*,7a*S*)-4-allyl-4-hydroxyhexahydrospiro[benzo[d][1,3]dioxole-2,1′-cyclohexane]-5,7-diyl)diacetamide (15): Synthesis of the corresponding diacetates was performed from **8** as described for **14**, producing **15** as a white foam (48 mg, 72% yield); $R_f = 0.28$ in 10% MeOH/CH_2Cl_2; ^{1}H NMR (500 MHz, $CDCl_3$). *Major isomer:* $\delta = 6.71$ (d, $J = 5.5$ Hz, 1H), 6.46 (d, $J = 8.8$ Hz, 1H), 5.94–5.82 (m, 1H), 5.18–5.07 (m, 2H), 4.07–3.91 (m, 2H), 3.83 (dd, $J = 9.6$, 10.0 Hz, 1H), 3.36 (d, $J = 9.1$ Hz, 1H), 2.37 (d, $J = 7.3$ Hz, 2H), 2.27–2.19 (m, 1H), 2.00 (s, 3H), 1.97 (s, 3H), 1.64–1.34 ppm (m, 11H); ^{13}C NMR (63 MHz, $CDCl_3$): $\delta = 170.6$, 170.0, 132.5, 111.2, 80.5, 75.0, 73.3, 49.4, 48.2, 40.6, 36.4, 36.2, 34.3, 25.0, 23.8, 23.3, 23.0 ppm; HRMS-ESI: *m/z*: calcd for $C_{19}H_{30}N_2NaO_5$: 389.2052 $[M+Na]^+$, found: 389.2045.

Synthesis of *N,N′*-((3a*R*,5*S*,7*R*,7a*S*)-4-(but-3-enyl)-4-hydroxyhexahydrospiro[benzo[d][1,3]dioxole-2,1′-cyclohexane]-5,7-diyl)diacetamide (16): Synthesis of the corresponding diacetates was performed from **9** as described for **14**, producing **16** as a white foam (36.3 mg, 50%); $R_f = 0.28$ in 10% MeOH/CH_2Cl_2; ^{1}H NMR ($CDCl_3$, 500 MHz): $\delta = 5.84$–5.74 (m, 1H), 5.01 (d, $J = 17.0$ Hz, 1H), 4.91 (d, $J = 10.5$ Hz, 1H), 4.02 (d, $J = 10.0$ Hz, 1H), 3.95 (br s, 2H), 3.44 (d, $J =$ 7.5 Hz, 1H), 2.30–2.20 (m, 1H), 2.15–2.05 (m, 1H), 1.97 (s, 3H), 1.94 (s, 3H), 1.94–1.88 (m, 1H), 1.90–1.80 (m, 2H), 1.63 (br s, 8H), 1.50–1.36 ppm (m, 2H); ^{13}C NMR (CD_3OD, 125 MHz): $\delta = 171.2$, 158.9, 137.5, 115.8, 112.9, 82.4, 78.5, 75.7, 56.3, 47.8, 37.1, 36.5, 33.2, 25.3, 24.1, 23.9, 23.3, 18.7 ppm; HRMS-ESI: *m/z*: calcd for $C_{20}H_{32}N_2NaO_5$: 403.2209 $[M+Na]^+$, found: 403.2204.

Allylation of the tertiary alcohols 14 and synthesis of *N,N′*-((3a*R*,5*S*,7*R*,7a*S*)-4-(allyloxy)-4-vinylhexahydrospiro[benzo[d][1,3]dioxole-2,1′-cyclohexane]-5,7-diyl)diacetamide (17): Alcohol **14** (0.23 g, 0.64 mmol) was dissolved in DMF (7.0 mL) and cooled to 0 °C. NaH (31 mg, 0.77 mmol, 60% *w/w*) was added, and the solution was stirred at 0 °C for 45 min. Freshly distilled allyl bromide (72 μL, 0.83 mmol) was added in one portion, and stirring was continued at RT for 24 h. A sat. aq solution of NH_4Cl (4.5 mL) was then added, and the mixture was extracted with CH_2Cl_2 (3×10 mL). The organic solution was dried ($MgSO_4$) and concentrated in vacuo. The crude product was purified by flash column chromatography on silica gel ($CH_2Cl_2 \rightarrow 10\%$ MeOH/CH_2Cl_2) to furnish alkenes **17a** (76 mg) and **17b** (46 mg) as white solids (122 mg, 48% overall yield); *Major isomer* (**17a**): $R_f = 0.69$ in 10% MeOH/CH_2Cl_2; ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 5.90$–5.85 (m, 3H), 5.78 (dd, $J = 11.3$, 17.8 Hz, 1H), 5.58 (d, $J = 17.5$ Hz, 1H), 5.40 (d, $J = 11.0$ Hz), 5.21 (d, $J =$ 17.0 Hz, 1H), 5.12 (d, $J = 10.0$ Hz, 1H), 4.36 (dd, $J = 5.3$, 13.3 Hz, 1H), 4.12 (dd, $J = 5.0$, 13.5 Hz, 1H), 4.02–3.98 (m, 2H), 3.90 (t, $J = 9.8$ Hz, 1H), 3.68 (d, $J = 9.0$ Hz, 1H), 2.70–2.40 (m, 1H), 1.96 (m, 6H), 1.67–1.44 ppm (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 170.0$, 169.3, 135.8, 134.7, 120.1, 115.6, 111.8, 81.8, 78.9, 75.6, 67.1, 52.4, 48.8, 36.7, 36.2, 34.4, 25.1, 23.8, 23.8, 23.6, 23.4 ppm; IR (neat): $\tilde{\nu} = 3230$, 2934, 1636, 1541, 1373, 1116, 1083, 1037, 914 cm^{-1}; HRMS-ESI: *m/z*: calcd for $C_{21}H_{33}N_2O_5$: 393.2384 $[M+H]^+$, found: 393.2384. *Minor isomer* (**17b**): $R_f = 0.61$ in 10% MeOH/CH_2Cl_2; ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 6.56$ (br s, 1H), 5.92–5.79 (m, 3H), 5.62–5.48 (m, 2H), 5.23 (d, $J = 15.5$ Hz, 1H), 5.05 (d, $J = 10.5$ Hz, 1H), 4.23 (m, 1H), 4.14 (dd, $J = 4.5$, 13.5 Hz, 1H), 4.04–4.02 (m, 2H), 3.74–3.66 (m, 2H), 2.30 (d, $J = 11.5$ Hz, 1H), 1.95 (s, 3H), 1.94 (s, 3H), 1.60–1.30 ppm (m, 11H); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 170.4$, 169.9, 135.5, 131.1, 121.7, 115.1, 111.9, 81.9, 80.0, 77.1, 65.5, 51.7, 48.6, 36.5, 36.2, 35.2, 25.1, 23.9, 23.7, 23.5, 23.4 ppm; IR (neat): $\tilde{\nu} = 3284$, 2937, 1653, 1558, 1373, 1131, 1083, 917 cm^{-1}; HRMS-ESI: *m/z*: calcd for $C_{21}H_{33}N_2O_5$: 393.2384 $[M+H]^+$, found: 393.2383.

Allylation of tertiary alcohols 15 and synthesis of *N,N′*-((3a*R*,5*S*,7*R*,7a*S*)-4-allyl-4-(allyloxy)hexahydrospiro[benzo[d][1,3]dioxole-2,1′-cyclohexane]-5,7-diyl)diacetamide (18): Similarly to **17**, diene **18** was isolated as a white solid that contained both epimers (at the quaternary center) in a ratio of 4:1 (58 mg, 90% yield); $R_f = 0.31$ in 10% MeOH/CH_2Cl_2; ^{1}H NMR (500 MHz, $CDCl_3$). *Major isomer:* $\delta = 7.40$ (d, $J = 7.4$ Hz, 1H; N*H*), 7.28 (d, $J = 6.6$ Hz, 1H; N*H*), 5.86 (m, 2H), 5.43–5.16 (m, 4H), 4.65 (dd, $J = 4.2$, 12.8 Hz, 1H), 4.36–4.29 (m, 1H), 4.25–4.13 (m, 1H), 4.09–3.93 (m, 2H), 3.56 (d, $J = 9.1$ Hz, 1H), 2.89 (dd, $J = 8.5$, 12.8 Hz, 1H), 2.49–2.39 (m, 1H), 2.36–2.27 (m, 1H), 2.08 (s, 3H), 2.07 (s, 3H), 1.84–1.49 ppm (m, 11H); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 169.8$, 169.0, 135.7, 135.3, 132.0, 129.1, 125.4, 120.0, 115.4, 111.6, 80.7, 77.9, 75.4, 65.4, 49.1, 48.9, 36.8, 36.3, 36.0, 34.8, 34.3, 25.1, 23.9, 23.8, 23.5, 23.4 ppm; HRMS-ESI: *m/z*: calcd for $C_{22}H_{34}N_2NaO_5$: 429.2365 $[M+Na]^+$, found: 429.2362.

Allylation of tertiary alcohols 16 and synthesis of *N,N′*-((3a*R*,5*S*,7*R*,7a*S*)-4-(allyloxy)-4-(but-3-enyl)hexahydrospiro[benzo[d][1,3]dioxole-2,1′-cyclohexane]-5,7-diyl)diacetamide (19): Similarly to **17**, dienes **19** were isolated as a white solid (both epimers at the quaternary center) in a ratio of 7:1 (50 mg, 70% yield); $R_f =$ 0.31 in 10% MeOH/CH_2Cl_2; ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 6.02$–5.82 (m, 2H), 5.32 (d, $J = 16.0$ Hz, 1H), 5.22 (d, $J = 9.0$ Hz, 1H), 5.14 (d, $J = 17.0$ Hz, 1H), 5.05 (d, $J = 12.5$ Hz, 1H), 4.65–4.46 (m, 1H), 4.30–4.13 (m, 2H), 4.09–3.90 (m, 2H), 3.60–3.48 (m, 1H), 2.47–2.32 (m, 1H), 2.30–2.10 (m, 5H), 2.05 (d, $J = 4.5$ Hz, 3H), 1.80–1.60 (m, 10H), 1.58–1.53 (m, 1H), 1.52–1.36 ppm (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 170.2$, 169.4, 138.4, 136.1, 135.7, 134.8, 117.3, 116.2, 115.7, 115.5, 115.0, 111.9, 81.5, 81.2, 78.2, 75.9, 75.7, 65.4, 65.3, 49.9, 49.7, 49.5, 49.2, 37.0, 36.8, 36.5, 36.4, 36.3, 34.7, 29.8, 28.6, 25.4, 24.0 ppm; HRMS-ESI: *m/z*: calcd for $C_{23}H_{36}N_2NaO_5$: 443.2522 $[M+Na]^+$; found: 443.2515.

Synthesis of (3a*R*,5*S*,7*R*,7a*S*)-4-allyl-5,7-diazidohexahydrospiro-[benzo[d][1,3]dioxole-2,1′-cyclohexan]-4-ol (20): At 0 °C, a biphasic mixture of NaN_3 (33 mg, 0.49 mmol) in H_2O (0.50 mL) and CH_2Cl_2 (0.50 mL) was treated with triflic anhydride (Tf_2O; 42 μL, 0.25 mmol) dropwise and allowed to warm to RT, with continuous stirring for another 2 h. A sat. aq solution of $NaHCO_3$ (0.60 mL) was added, and the mixture was extracted with CH_2Cl_2 (2×1 mL). This solution of TfN_3 was added dropwise to a mixture of the diamine (14 mg, 0.049 mmol) with $CuSO_4 \cdot 5H_2O$ (2.0 mg, 0.007 mmol) in MeOH (2 mL), H_2O (0.50 mL) and Et_3N (40 μL, 0.29 mmol) and allowed to stir vigorously for 20 h. The solvents were partially removed in vacuo (CAUTION: danger of explosion if the sample reaches complete dryness) and the crude product was purified by flash column chromatography on silica gel (hexanes→30% EtOAc/hexanes) to furnish diazides **20** (14 mg, 88% yield); $R_f = 0.68$ in 30% EtOAc/hexanes; ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 5.84$ (m, 1H),

5.26–5.19 (m, 2H), 3.92 (t, J=9.7 Hz, 1H), 3.54 (td, J=4.2, 11.0 Hz, 1H), 3.19 (d, J=9.7 Hz, 1H), 3.13 (dd, J=4.3, 11.0 Hz, 2H), 2.59–2.48 (m, 2H), 2.21 (m, 1H), 1.96 (q, J=11.9 Hz, 1H), 1.73–1.51 ppm (m, 10H); ^{13}C NMR (125 MHz, $CDCl_3$): δ=131.6, 120.6, 112.8, 79.1, 76.0, 73.9, 59.6, 57.8, 40.5, 36.3, 35.9, 30.8, 24.9, 23.6 ppm; IR (neat): $\tilde{\nu}$=3436, 2937, 2861, 2098, 1264, 1115, 921, 736 cm^{-1}.

Allylation of tertiary alcohols 20 and synthesis of (3a*R*,5*S*,7*R*,7a*S*)-4-allyl-4-(allyloxy)-5,7-diazidohexahydrospiro-[benzo[d][1,3]dioxole-2,1′-cyclohexane] (21): At 0 °C, a solution of homoallylic alcohol **20** (56 mg, 0.17 mmol) in dry DMF (3.5 mL) was treated with NaH (9.4 mg, 0.23 mmol, 60% *w/w*), and the mixture was allowed to stir for 15 min. This reaction mixture was cooled to −10 °C, then allyl bromide (23 μL, 0.27 mmol) was added dropwise and the temperature was slowly increased to 0 °C over 3 h. Sat. aq NH_4Cl (4 mL) was added, and the mixture was extracted with EtOAc (3×10 mL), the organic solution dried ($MgSO_4$), and the solvents removed in vacuo. The crude product was purified by flash column chromatography on silica gel (hexanes→20% EtOAc/hexanes) to furnish the diazide **21** (55 mg, 88% yield); R_f=0.53 in 10% EtOAc/hexanes; ^{1}H NMR (500 MHz, $CDCl_3$): δ=5.88 (m, 1H), 5.76 (m, 1H), 5.28 (d, J=10.3 Hz, 1H), 5.24 (d, J=10.0 Hz, 1H), 5.18 (d, J=10.0 Hz, 1H), 5.13 (dd, J=1.4, 10.3 Hz, 1H), 4.45 (dd, J=5.0, 13.0 Hz, 1H), 3.96 (t, J=9.7 Hz, 1H), 3.51 (m, 1H), 3.24 (d, J=9.7 Hz, 1H), 2.93 (dd, J=5.0, 11.0 Hz, 1H), 2.88 (dd, J=7.0, 13.0 Hz, 1H), 2.61 (dd, J=8.2, 13.0 Hz, 1H), 2.16–2.09 (m, 2H), 1.64–1.41 ppm (m, 10H); ^{13}C NMR (62 MHz, $CDCl_3$): δ=134.9, 131.8, 120.4, 115.4, 112.4, 79.3, 78.3, 76.3, 65.2, 58.8, 58.5, 36.2, 34.4, 30.2, 24.9, 23.7 ppm; IR (neat): $\tilde{\nu}$=2936, 2099, 1253, 1113, 916, 848 cm^{-1}; HRMS-ESI: *m/z*: calcd for $C_{18}H_{27}N_6O_3$: 375.2139 $[M+H]^+$, found: 375.2136.

Ring-closing metathesis for the synthesis of *N*,*N*′-((2′*S*,3a*R*,5*S*,7*R*,7a*S*)-2,2-cyclohexyl-4′,5,6,7,7a,7′-hexahydro-3a*H*,3′*H*-spiro[benzo[d][1,3]dioxole-4,2′-oxepine]-5,7-diyl)diacetamide (22): Grubbs' second-generation catalyst (16 mg, 0.019 mmol) was added to a stirred solution of diene **17a** (76 mg, 0.19 mmol) in CH_2Cl_2 (39 mL) at RT, and the solution was allowed to stir for 24 h (the color changed from green to dark brown). The solvent was removed in vacuo and the crude product was purified by flash chromatography on silica gel (CH_2Cl_2→10% MeOH/CH_2Cl_2) to furnish spiro ether **22** (62 mg, 88% yield) as a light-brown solid; R_f=0.31 in 7% MeOH/CH_2Cl_2; $[\alpha]_D^{22}$=−34.0° (c=0.5, CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$): δ=6.21 (brs, 1H), 6.06 (d, J=6.0 Hz, 1H), 5.85 (d, J=9.0 Hz, 1H), 5.57 (d, J=6.0 Hz, 1H), 4.70 (d, J=12.5 Hz, 1H), 4.65 (d, J=12.5 Hz, 1H), 4.28 (m, 1H), 4.03 (m, 1H), 3.88 (t, J=10.0 Hz, 1H), 3.50 (d, J=9.0 Hz, 1H), 2.34 (m, 1H), 1.98 (s, 3H), 1.91 (s, 3H), 1.60–1.24 ppm (m, 11H); ^{13}C NMR (125 MHz, $CDCl_3$): δ=170.4 (2C), 129.4, 127.2, 111.6, 92.2, 80.7, 76.9, 75.8, 49.7, 48.3, 36.6, 35.9, 34.7, 25.1, 23.7 (2C), 23.2, 22.9 ppm; IR (neat): $\tilde{\nu}$=3278, 2937, 1652, 1558, 1374, 1117, 1036 cm^{-1}; HRMS-ESI: *m/z*: calcd for $C_{19}H_{29}N_2O_5$: 365.2071 $[M+H]^+$, found: 365.2073.

Ring-closing metathesis for the synthesis of *N*,*N*′-((2′*R*,3a*R*,5*S*,7*R*,7a*S*)-2,2-cyclohexyl-5,6,7,7a-tetrahydro-3a*H*,5′*H*-spiro[benzo[d][1,3]dioxole-4,2′-furan]-5,7-diyl)diacetamide (25): Grubbs' second-generation catalyst (9.5 mg, 0.011 mmol) was added to a stirred solution of diene **17b** (44 mg, 0.11 mmol) in CH_2Cl_2 (23 mL) at RT, and the solution was allowed to stir for 18 h (color change from green to dark brown). The solvent was removed in vacuo and the crude product was purified by flash chromatography on silica gel with a gradient elution system (CH_2Cl_2→10% MeOH/CH_2Cl_2) to furnish spiro ether **25** (28 mg, 69% yield) as a light-brown solid; R_f=0.32 in 7% MeOH/CH_2Cl_2; $[\alpha]_D^{22}$=−12.9° (c=1.0, CH_2Cl_2); ^{1}H NMR (500 MHz, CD_3OD): δ=7.33 (brs, 1H), 6.53 (brs, 1H), 6.31 (d, J=5.5 Hz, 1H), 5.78 (m, 1H), 4.65 (d, J=13.0 Hz, 1H), 4.52 (d, J=13.0 Hz, 1H), 4.17 (m, 1H), 4.05 (m, 1H), 3.61 (m, 2H), 2.29 (m, 1H), 1.95 (s, 3H), 1.91 (s, 3H), 1.58–1.53 (m, 8H), 1.39–1.33 ppm (m, 3H); ^{13}C NMR (125 MHz, CD_3OD): δ=170.8, 170.7, 134.5, 122.5, 112.3, 94.0, 82.0, 77.8, 75.8, 52.6, 48.6, 36.7, 36.5, 36.0, 27.4, 25.4, 24.0, 23.8, 23.7 ppm; IR (neat): $\tilde{\nu}$=3274, 2937, 1635, 1548, 1374, 1067, 1040, 914 cm^{-1}; HRMS-ESI: *m/z*: calcd for $C_{19}H_{29}N_2O_5$: 365.2071 $[M+H]^+$, found: 365.2069.

Ring-closing metathesis for the synthesis of *N*,*N*′-((2′*S*,3a*R*,5*S*,7*R*,7a*S*)-2,2-cyclohexyl-3′,5,6,6′,7,7a-hexahydro-3a*H*-spiro[benzo[d][1,3]dioxole-4,2′-pyran]-5,7-diyl)diacetamide (23) and its 2′*R* isomer (26): According to the synthesis of **22** and **25**, spiro-olefins **23** (18 mg) and **26** (3.0 mg) were isolated from dienes **18** in 70% overall yield; *Major isomer* (**23**): R_f=0.46 in 10% MeOH/CH_2Cl_2; $[\alpha]_D^{22}$=−12.0° (c=0.2, CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$): δ=5.94–5.79 (m, 2H), 5.76 (d, J=6.6 Hz, 1H), 5.71 (d, J=9.9 Hz, 1H), 4.60 (d, J=15.4 Hz, 1H), 4.09 (d, J=15.4 Hz, 1H), 4.04–3.86 (m, 3H), 3.38 (d, J=9.13 Hz, 1H), 2.39–2.24 (m, 2H), 1.98 (brs, 6H), 1.70–1.36 ppm (m, 12H); ^{13}C NMR (125 MHz, $CDCl_3$): δ=169.9, 169.7, 124.6, 122.4, 111.8, 85.3, 74.8, 72.5, 64.0, 51.7, 49.0, 36.7, 36.1, 34.1, 28.6, 25.1, 23.9, 23.8, 23.6, 23.4 ppm; IR (neat): $\tilde{\nu}$=3259, 2934, 2859, 1635, 1545, 1371, 1126, 1080, 733 cm^{-1}; HRMS-ESI: *m/z*: calcd for $C_{20}H_{30}N_2NaO_7$: 401.2052 $[M+Na]^+$, found 401.2050; *Minor isomer* (**26**): R_f=0.29 in 10% MeOH/CH_2Cl_2; $[\alpha]_D^{22}$=+3.0° (c=0.2, CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$): δ=6.09 (d, J=7.8 Hz, 1H), 6.01 (d, J=6.1 Hz, 1H), 4.31 (m, 2H), 4.17 (m, 1H), 3.92 (m, 1H) 3.62–3.52 (m, 2H), 2.57 (d, J=18.0 Hz, 1H), 2.41 (m, 1H), 2.08 (d, J=18.0 Hz, 1H), 1.96 (s, 3H), 1.90 (s, 3H), 1.74–1.53 (m, 8H), 1.36 ppm (m, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ=170.4, 169.8, 126.4, 122.0, 111.8, 83.5, 75.7, 73.8, 63.1, 54.3, 48.4, 36.4, 36.1, 35.8, 25.0, 23.6, 23.6, 23.4, 23.1, 22.1 ppm; IR (neat): $\tilde{\nu}$=3287, 2935, 2861, 1653, 1558, 1448, 1375, 1133, 1089 cm^{-1}; HRMS-ESI (*m/z*) calcd for $C_{20}H_{30}N_2NaO_7$: 401.2052 $[M+Na]^+$, found: 401.2056.

Ring-closing metathesis for the synthesis of *N*,*N*′-((2′*S*,3a*R*,5*S*,7*R*,7a*S*)-2,2-cyclohexyl-5,6,7,7a-tetrahydro-3a*H*,5′*H*-spiro[benzo[d][1,3]dioxole-4,2′-furan]-5,7-diyl)diacetamide (24): According to the synthesis of **22** and **25**, spiro-olefin **24** (17 mg, 60% yield) was isolated as a dark-yellow solid from dienes **19**. The minor isomer could not be isolated due to its very small amount; R_f=0.25 in 10% MeOH/CH_2Cl_2; ^{1}H NMR (500 MHz, $CDCl_3$): δ=6.19 (d, J=9.5 Hz, 1H), 5.97 (d, J=7.0 Hz, 1H), 5.81 (brs, 1H), 5.72 (brs, 1H), 4.17 (dd, J=5.0, 16.0 Hz, 2H), 4.13–4.06 (m, 1H), 4.00 (t, J=10.0 Hz, 1H), 3.83 (dd, J=6.5, 13.5 Hz, 1H), 3.50 (d, J=9.0 Hz, 1H), 2.78 (appt, J=13.0 Hz, 1H), 2.65 (appt, J=14.0 Hz, 1H), 2.45–2.35 (m, 1H), 2.34–2.28 (m, 2H), 2.08 (s, 3H), 2.07 (s, 3H), 1.98–1.94 (dd, J=4.0, 15.0 Hz, 1H), 1.88–1.80 (m, 1H), 1.80–1.62 (m, 8H), 1.63–1.54 ppm (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$): δ=170.2, 169.4, 131.4, 129.2, 111.8, 84.2, 75.4, 64.5, 54.0, 49.0, 36.9, 36.4, 35.8, 33.8, 26.3, 25.4, 24.1, 23.9, 23.8 ppm; HRMS-ESI: *m/z*: calcd for $C_{21}H_{32}N_2NaO_5$: 415.2209 $[M+Na]^+$, found: 415.2205.

Cleavage of the ketal and synthesis of *N*,*N*′-((5*S*,6*S*,8*R*,9*S*,10*R*)-9,10-dihydroxy-1-oxaspiro[4.5]dec-3-ene-6,8-diyl)diacetamide (27): A solution of spiro-olefin **22** (30 mg, 0.082 mmol) in a mixture of HOAc/THF/H_2O (3:3:1 *v/v/v*, 17 mL) was heated to 45 °C for 3 h. The solvent was removed in vacuo, and the crude product was purified by flash chromatography on silica gel (5→20% MeOH/CH_2Cl_2) to furnish diol **27** (22 mg, 94% yield) as a white solid; R_f=0.23 in 15% MeOH/CH_2Cl_2; $[\alpha]_D^{22}$=−51.7° (c=1.0, MeOH); ^{1}H NMR (500 MHz, CD_3OD): δ=7.80 (d, J=9.5 Hz, 1H), 6.02 (d, J=6.0 Hz, 1H), 5.52 (m, 1H), 4.71–4.64 (m, 2H), 4.06 (m, 1H), 3.77 (m, 1H), 3.53 (t, J=10.0 Hz, 1H), 3.42 (d, J=9.5 Hz, 1H), 1.95 (s, 3H), 1.86 (s, 3H), 1.81 (dt, J=4.4, 12.4 Hz, 1H), 1.72 ppm (q, J=12.4 Hz, 1H);

^{13}C NMR (125 MHz, CD_3OD): δ = 171.9, 171.2, 128.5, 127.6, 95.2, 76.6, 75.3, 73.3, 50.2, 49.2, 32.0, 21.3, 21.0 ppm; IR (neat): $\tilde{\nu}$ = 3267, 2932, 1627, 1546, 1373, 1084, 1018, 741 cm^{-1}; HRMS-ESI: *m/z*: calcd for $C_{13}H_{21}N_2O_5$: 285.1445 $[M+H]^+$, found: 285.1444.

Cleavage of the ketal and synthesis of *N,N'*-((6*S*,7*S*,9*R*,10*S*,11*R*)-10,11-dihydroxy-1-oxaspiro[5.5]undec-3-ene-7,9-diyl)diacetamide (28): According to the synthesis of **27**, diol **28** was isolated as a white solid (28 mg, 95% yield); R_f = 0.63 in 30% MeOH/CH_2Cl_2; $[\alpha]_D^{22}$ = −54.4° (*c* = 0.36, MeOH); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 6.23 (d, *J* = 5.1 Hz, 1H), 6.00 (d, *J* = 4.7 Hz, 1H), 5.92–5.76 (m, 2H), 4.61 (d, *J* = 15.7 Hz, 1H), 4.10 (d, *J* = 15.5 Hz, 1H), 3.94–3.76 (m, 2H), 3.71 (dd, *J* = 9.5, 9.5 Hz, 1H), 3.47–3.67 (m, 1H), 2.30–2.15 (m, 2H), 1.99 (s, 3H), 1.98 (s, 3H), 1.88 (m, 1H), 1.70 ppm (ddd, *J* = 12.1, 12.1, 12.2 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 172.2, 170.1, 125.2, 123.6, 81.3, 74.0, 73.7, 63.8, 51.8, 51.1, 32.5, 28.5, 23.5, 23.4 ppm; IR (neat): $\tilde{\nu}$ = 3285, 3078, 2927, 1696, 1635, 1558, 1541, 1086, 764 cm^{-1}; HRMS-ESI: *m/z*: calcd for $C_{14}H_{22}N_2NaO_5$: 321.1426 $[M+Na]^+$, found: 321.1421.

Cleavage of the ketal and synthesis of *N,N'*-((5*R*,6*S*,8*R*,9*S*,10*R*)-9,10-dihydroxy-1-oxaspiro[4.5]dec-3-ene-6,8-diyl)diacetamide (30): According to the synthesis of **27**, diol **30** (17 mg, 73% yield) was isolated as a white solid; R_f = 0.18 in 20% MeOH/CH_2Cl_2; ^{1}H NMR (500 MHz, CD_3OD): δ = 6.19 (d, *J* = 6.5 Hz, 1H), 5.63 (m, 1H), 4.55 (d, *J* = 12.6 Hz, 1H), 4.39 (d, *J* = 12.6 Hz, 1H), 4.01 (dd, *J* = 3.9, 13.2 Hz, 1H), 3.71 (m, 1H), 3.38 (d, *J* = 9.7 Hz, 1H), 3.26 (t, *J* = 10.0 Hz, 1H), 1.84 (s, 3H), 1.82 (m, 1H), 1.69 (s, 3H), 1.33 ppm (q, *J* = 12.8 Hz, 1H); ^{13}C NMR (63 MHz, CD_3OD): δ = 173.4, 173.0, 134.5, 123.7, 97.5, 79.1, 77.1, 75.3, 52.2, 52.1, 34.5, 23.0, 22.9 ppm; HRMS-ESI: *m/z*: calcd for $C_{13}H_{20}N_2NaO_5$: 307.1270 $[M+Na]^+$, found: 307.1272.

Cleavage of the ketal and synthesis of *N,N'*-((6*R*,7*S*,9*R*,10*S*,11*R*)-10,11-dihydroxy-1-oxaspiro[5.5]undec-3-ene-7,9-diyl)diacetamide (31): According to the synthesis of **27**, diol **31** was isolated as a white solid (7.0 mg, 85% yield); R_f = 0.18 in 20% MeOH/CH_2Cl_2; $[\alpha]_D^{22}$ = −2.8° (*c* = 0.7, MeOH); ^{1}H NMR (250 MHz, CD_3OD): δ = 5.86 (m, 1H), 5.76 (m, 1H), 4.23 (m, 2H), 4.18 (dd, *J* = 4.4, 12.8 Hz, 1H), 3.75 (m, 1H), 3.37 (d, *J* = 9.9 Hz, 1H), 3.23 (d, *J* = 9.9 Hz, 1H), 2.54 (dt, *J* = 2.7, 18.0 Hz, 1H), 2.06 (m, 1H), 1.94 (s, 3H), 1.87 (s, 3H), 1.83 (dt, *J* = 4.6, 13.0 Hz, 1H), 1.46 ppm (q, *J* = 13.0 Hz, 1H); ^{13}C NMR (63 MHz, CD_3OD): δ = 175.4, 172.6, 126.5, 123.8, 80.47, 76.7, 73.7, 63.9, 54.2, 51.8, 34.8, 23.1, 22.9, 22.6 ppm; IR (neat): $\tilde{\nu}$ = 3289, 2921, 1643, 1553, 1095, 1036 cm^{-1}.

Synthesis of (5*S*,6*R*,7*S*,8*R*,10*S*)-8,10-diamino-1-oxaspiro[4.5]decane-6,7-diol (32): Palladium on activated C (10 wt%, 2.0 mg, 0.0015 mmol) was added to a solution of **27** (8.5 mg, 0.030 mmol) in EtOAc/MeOH (2.2 mL, 10:1, *v/v*). The suspension was stirred under an H_2 atmosphere at RT for 18 h and then filtered trough Celite. The solvent was evaporated and the hydrogenated diacetate (8.5 mg, quant.) was obtained without further purification as a white solid; R_f = 0.49 in 25% MeOH/CH_2Cl_2; $[\alpha]_D^{22}$ = −20.9° (*c* = 0.8, MeOH); ^{1}H NMR (500 MHz, CD_3OD): δ = 3.99–3.92 (m, 3H), 3.72 (td, *J* = 4.0, 12.0 Hz, 1H), 3.49 (t, *J* = 10.0 Hz, 1H), 3.25 (d, *J* = 9.5 Hz, 1H), 2.12 (m, 1H), 1.94 (m, 7H), 1.80–1.71 ppm (m, 3H), 1.66 ppm (q, *J* = 12.3 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD): δ = 171.8, 171.3, 86.4, 77.5, 73.6, 70.3, 50.3 (2C), 32.9, 30.5, 26.2, 21.3, 21.0 ppm; IR (neat): $\tilde{\nu}$ = 3276, 1653, 1559, 1375, 1086 cm^{-1}.

A solution of the diacetate (8.5 mg, 0.030 mmol) and KOH (0.12 g, 2.1 mmol) in H_2O (2.3 mL) was heated to 140 °C for 48 h. The solvent was removed in vacuo, and the crude product was purified by flash chromatography on silica gel (MeOH→20% NH_4OH/MeOH) to obtain spiro ether **32** as a white solid (1.3 mg, 21%); R_f = 0.62 in 25% MeOH/CH_2Cl_2; $[\alpha]_D^{22}$ = +11.8° (*c* = 0.12, MeOH); ^{1}H NMR (500 MHz, D_2O): δ = 3.87 (m, 2H), 3.40 (t, *J* = 10.0 Hz, 1H), 3.27 (d, *J* = 9.5 Hz, 1H), 2.95–2.92 (m, 2H), 2.09 (m, 1H), 1.96–1.88 (m, 3H), 1.80 (m, 1H), 1.52 ppm (q, *J* = 12.5 Hz, 1H); ^{13}C NMR (125 MHz, D_2O): δ = 86.4, 76.4, 73.1, 71.6, 51.9, 50.7, 32.5, 30.8, 26.0 ppm; IR (neat): $\tilde{\nu}$ = 3258, 2890, 1635, 1540, 1100, 616 cm^{-1}; HRMS-ESI: *m/z*: calcd for $C_9H_{19}N_2O_3$: 203.1390 $[M+H]^+$, found: 203.1391.

Synthesis of *N*-((6*S*,7*S*,9*R*,10*S*,11*R*)-9-amino-10,11-dihydroxy-1-oxaspiro[5.5]undecan-7-yl)acetamide (33): According to the sequence described for **32**, 6-membered spirocyclic analogue was isolated as a colorless solid (8.6 mg, 73% yield); R_f = 0.41 in 15% MeOH/CH_2Cl_2; $[\alpha]_D^{22}$ = −6.8° (*c* = 0.5, MeOH); ^{1}H NMR (500 MHz, CD_3OD): δ = 4.27 (m, 1H), 3.74–3.68 (m, 2H), 3.61 (t, *J* = 9.8 Hz, 1H), 3.34–3.27 (m, 2H), 2.21–2.10 (m, 2H), 1.97 (s, 3H), 1.94 (s, 3H), 1.78–1.50 ppm (m, 6H); ^{13}C NMR (62.9 MHz, CD_3OD): δ = 171.9, 171.4, 81.6, 74.4, 72.7, 64.0, 51.5, 50.5, 31.9, 29.1, 24.7, 21.3, 21.1, 19.7 ppm; IR (neat): $\tilde{\nu}$ = 3289, 2940, 1637, 1431, 1204, 1138, 1059, 723 cm^{-1}; HRMS-ESI: *m/z*: calcd for $C_{14}H_{24}N_2NaO_5$: 323.15774 $[M+Na]^+$, found: 323.15754.

A solution of the acetate (7 mg, 0.02 mmol) and KOH (92 mg, 1.6 mmol) in H_2O (1.7 mL) was heated in a sealed pressurized tube to 140 °C for 16 h, and afterwards the solvent was evaporated. The crude product was purified by column chromatography on silica gel (MeOH→25% NH_4OH/MeOH) to obtain amine **33** as a colorless solid. (2.0 mg, 40%); R_f = 0.48 in 25% NH_4OH/MeOH; $[\alpha]_D^{22}$ = −6.9° (*c* = 0.072, H_2O); ^{1}H NMR (500 MHz, D_2O): δ = 4.07 (m, 1H), 3.86 (m, 1H), 3.76 (dd, *J* = 4.5, 17.0 Hz, 1H), 3.55 (t, *J* = 9.5 Hz, 1H), 3.35 (m, 1H), 2.85 (m, 1H), 2.07 (s, 3H), 1.78–1.57 ppm (m, 8H); ^{13}C NMR (125 MHz, D_2O): δ = 166.1, 80.3, 76.1, 74.7, 65.4, 53.0, 51.8, 32.6, 29.4, 24.7, 22.7, 19.8 ppm; IR (neat): $\tilde{\nu}$ = 3372, 2934, 1653, 1588, 1472, 1356, 1371 1083, 1056 cm^{-1}; HRMS-ESI: *m/z*: calcd for $C_{12}H_{23}N_2O_4$: 259.16523 $[M+H]^+$, found: 259.16507.

Synthesis of (5*S*,6*R*,7*S*,8*R*,10*S*)-8,10-diamino-1-oxaspiro[4.5]dec-3-ene-6,7-diol (34): A solution of **27** (10 mg, 0.037 mmol) and KOH (0.14 g, 2.6 mmol) in H_2O (2.8 mL) was heated to 140 °C for 48 h. The solvent was removed in vacuo and the crude product was purified by flash chromatography on silica gel (MeOH→17% NH_4OH/MeOH) to obtain spiro ether **34** as a white solid. (3.6 mg, 50% yield): R_f = 0.48 in 25% NH_4OH/MeOH; $[\alpha]_D^{22}$ = +6.5° (*c* = 0.34, MeOH); ^{1}H NMR (500 MHz, D_2O): δ = 6.15 (d, *J* = 5.5 Hz, 1H), 5.53 (m, 1H), 4.61 (m, 2H), 3.38 (d, *J* = 9.5 Hz, 1H), 3.29 (t, *J* = 10.0 Hz, 1H), 2.90 (dd, *J* = 4.0, 12.5 Hz, 1H), 2.74 (m, 1H), 1.88 (m, 1H), 1.37 ppm (q, *J* = 12.5 Hz, 1H); ^{13}C NMR (62.5 MHz, D_2O): δ = 131.1, 126.5, 95.6, 77.7, 75.0, 74.5, 50.6, 50.5, 33.7 ppm; IR (neat): $\tilde{\nu}$ = 3348, 2860, 1570, 1472, 1379, 1109, 1076, 990, 744 cm^{-1}; HRMS-ESI: *m/z*: calcd for $C_9H_{17}N_2O_3$: 201.1234 $[M+H]^+$, found: 201.1236.

Synthesis of (6*S*,7*R*,8*S*,9*R*,11*S*)-9,11-diamino-1-oxaspiro[5.5]undec-3-ene-7,8-diol (35): A solution of the acetate **28** (21 mg, 0.070 mmol) and KOH (0.27 g, 4.9 mmol) in H_2O (5.0 mL) was heated in a sealed tube at 150 °C for 24 h, then the solvent was evaporated. The crude product was purified by column chromatography on silica gel (MeOH→25% NH_4OH/MeOH) to obtain diamine **35** as a colorless solid. (7.0 mg, 47%); R_f = 0.57 in 25% NH_4OH/MeOH; $[\alpha]_D^{22}$ = −2.0° (*c* = 0.5, H_2O); ^{1}H NMR (500 MHz, D_2O): δ = 5.83 (m, 2H), 4.33 (d, *J* = 15.5 Hz, 1H), 4.14 (d, *J* = 15.0 Hz, 1H), 3.72 (t, *J* = 10.0 Hz, 1H), 3.27 (d, *J* = 9.0 Hz, 1H), 3.19 (m, 1H), 3.08 (m, 1H), 2.33 (d, *J* = 18.0 Hz, 1H), 2.17 (d, *J* = 17.5 Hz, 1H), 2.06 (m, 1H), 1.92 ppm (m, 1H); ^{13}C NMR (125 MHz, D_2O): δ = 126.3, 122.3, 77.9, 73.0, 69.7, 63.3, 53.3, 50.5, 27.6, 27.0 ppm; IR (neat): $\tilde{\nu}$ = 3245,

1626, 1525, 1437, 1202, 1086, 616 cm^{-1}; HRMS-ESI: *m/z*: calcd for $C_{10}H_{19}N_2O_3$: 215.13902 $[M+H]^+$, found: 215.13890.

Cleavage of the ketal and hydrolysis for the synthesis of (1*R*,2*S*,3*R*,5*S*,6*S*)-3,5-diamino-7-oxaspiro[5.6]dodec-9-ene-1,2-diol (36): According to the synthesis of **27**, diol **29** (28 mg, 75% yield, $R_f = 0.48$ in 10% MeOH/CH_2Cl_2) was isolated as a white solid and immediately used for the next step. According to the hydrolysis step described for **32**, 7-membered unsaturated analogue **36** was isolated after flash column chromatography on silica gel (MeOH→30% NH_4OH/MeOH) as a white solid (14 mg, 53% yield); $R_f = 0.73$ in 10% NH_4OH/MeOH; $[\alpha]_D^{22} = +0.7^\circ$ ($c = 1.1$, MeOH); ^1H NMR (D_2O; 500 MHz): $\delta = 5.74$ (brs, 1H), 5.60 (brs, 1H), 4.31 (d, $J = 16.4$ Hz, 1H), 4.21 (d, $J = 16.4$ Hz, 1H), 4.04 (brs, 1H), 3.87 (brs, 1H), 3.73 (brs, 1H), 3.55 (brs, 1H), 3.46 (brs, 1H), 3.28 (brs, 1H), 2.20 (brs, 1H), 2.15 (d, $J = 14.5$ Hz, 2H), 2.07–2.03 (m, 1H), 1.95–1.89 (m, 1H), 1.86–1.81 (m, 2H), 1.53 ppm (brs, 1H); ^{13}C NMR (D_2O, 125 MHz): $\delta = 131.8$, 128.3, 78.4, 73.1, 72.3, 60.5, 48.7, 47.3, 33.0, 23.6, 20.8 ppm; HRMS-ESI: *m/z*: calcd for $C_{11}H_{20}N_2O_3$: 228.1474 $[M+H]^+$, found: 228.1471.

Synthesis of (1*R*,2*S*,3*R*,5*S*,6*S*)-3,5-diamino-7-oxaspiro[5.6]dodecane-1,2-diol (37): Hydrolysis of **29** followed by hydrogenation, according to the procedure described for **32**, produced 7-membered unsaturated analogue **37**, which was isolated after flash column chromatography on silica gel (MeOH→30% NH_4OH/MeOH) as a white amorphous solid. (21 mg, 69% yield); $R_f = 0.71$ in 10% NH_4OH/MeOH; $[\alpha]_D^{22} = -0.6^\circ$ ($c = 2.1$, MeOH); ^1H NMR (500 MHz, D_2O): $\delta = 4.12$ (t, $J = 11.6$ Hz, 1H), 3.65 (t, $J = 9.9$ Hz, 1H), 3.41–3.03 (m, 3H), 2.37–2.15 (m, 1H), 2.10–1.87 (m, 2H), 1.82 (brs, 2H), 1.78–1.74 (d, $J = 11.6$ Hz, 1H), 1.70–1.29 (m, 3H), 1.24–1.09 ppm (m, 2H); ^{13}C NMR (125 MHz, D_2O): $\delta = 77.2$, 76.8, 70.4, 67.6, 55.4, 50.7, 33.5, 31.7, 29.9, 28.0, 25.1 ppm; HRMS-ESI: *m/z*: calcd for $C_{11}H_{22}N_2O_3$: 230.1630 $[M+H]^+$, found: 230.1627.

Upjohn dihydroxylation of olefins 28 and synthesis of *N*,*N*′-((3*S*,4*R*,6*S*,7*S*,9*R*,10*S*,11*R*)-3,4,10,11-tetrahydroxy-1-oxaspiro[5.5]undecane-7,9-diyl)diacetamide (38) and its 3*R*,4*S*-isomer (39): *N*-Methylmorpholine *N*-oxide (11 mg, 0.094 mmol) was added to a solution of olefin **28** (10 mg, 0.033 mmol) in acetone/H_2O (1:1 (*v/v*), 2 mL), then OsO_4 (2.5 wt% in *t*BuOH, 0.035 mL, 3.4×10^{-3} mmol, 10 mol%) was added, and the solution was stirred at RT for 3 h. The solvent was removed in vacuo, and the crude residue was purified by flash column chromatography on silica gel (CH_2Cl_2→30% MeOH/CH_2Cl_2) to furnish tetraols **38** and **39** (1:9 *dr*; 9.8 mg, 90% overall yield); **38** (minor): ^1H NMR (500 MHz, CD_3OD): $\delta = 4.11$ (m, 1H), 4.06–3.94 (m, 2H), 3.90–3.74 (m, 2H), 3.71 (m, 1H), 3.44 (m, 2H), 2.23 (m, 1H), 2.09 (m, 1H), 1.99 (s, 3H), 1.98 (s, 3H), 1.81 (m, 1H), 1.61 ppm (m, 1H).

39 (major)**:** $R_f = 0.27$ in 30% MeOH/CH_2Cl_2; ^1H NMR (500 MHz, CD_3OD): $\delta = 4.50$ (m, 1H), 4.44 (d, $J = 11.8$ Hz, 1H), 3.83–3.69 (m, 5H), 3.59 (dd, $J = 9.9$, 9.9 Hz, 1H), 1.98 (s, 3H), 1.95 (s, 3H), 1.91 (m, 1H), 1.80 (dd, $J = 4.7$, 4.7 Hz, 1H), 1.71 (m, 1H), 1.63 ppm (dd, $J = 5.9$, 12.9 Hz, 1H).

Upjohn dihydroxylation of olefin 23 and synthesis of *N*,*N*′-((2′*S*,3a*R*,4′*S*,5*S*,5′*R*,7*R*,7a*S*)-2,2-cyclohexyl-4′,5′-dihydroxyoctahydro-3a*H*-spiro[benzo[d][1,3]dioxole-4,2′-pyran]-5,7-diyl)diacetamide (40): *N*-Methylmorpholine *N*-oxide (44 mg, 0.37 mmol), and then OsO_4 (2.5 wt% in *t*BuOH, 0.12 mL, 0.012 mmol) were added to a stirred solution of olefin **23** (47 mg, 0.12 mmol) in a mixture of acetone/H_2O (1:1 (*v/v*), 8 mL), and the solution was allowed to stir, at 0 °C, for 3 h. The solvent was removed in vacuo, and the crude product was purified by flash column chromatography on silica gel (CH_2Cl_2→30% MeOH/CH_2Cl_2) to furnish the white crystalline product **40** (40 mg, 78% yield); $R_f = 0.50$ in 20% MeOH/CH_2Cl_2; $[\alpha]_D^{22} = -32.0^\circ$ ($c = 0.7$, MeOH); ^1H NMR (500 MHz, $CDCl_3$): $\delta = 6.00$ (m, 1H), 5.52 (m, 1H), 4.41 (m, 1H), 4.28 (d, $J = 12.1$ Hz, 1H), 4.02 (m, 1H), 3.91–3.81 (m, 4H), 3.35 (d, $J = 9.3$ Hz, 1H), 2.25 (m, 1H), 2.02 (s, 3H), 1.98 (s, 3H), 1.80–1.38 ppm (m, 13H); ^{13}C NMR (63 MHz, CD_3OD): $\delta = 173.2$, 172.9, 112.6, 86.6, 79.9, 76.7, 69.6, 69.2, 67.0, 53.4, 37.6, 37.2, 34.4, 33.6, 26.2, 25.0, 22.8, 22.6 ppm; IR (neat): $\tilde{\nu} = 3272$, 2922, 2855, 1653, 1522, 1456, 1084, 801, 620 cm^{-1}; HRMS-ESI: *m/z*: calcd for $C_{20}H_{32}N_2NaO_7$: 435.2107 $[M+Na]^+$, found: 435.2111.

Synthesis of final product (3*S*,4*R*,6*S*,7*R*,8*S*,9*R*,11*S*)-9,11-diamino-1-oxaspiro[5.5]undecane-3,4,7,8-tetraol (2): The tetraol diacetamide **39** (6.0 mg, 0.024 mmol) was dissolved in H_2O (2.0 mL) and KOH(s) (0.10 g, 1.7 mmol) was added. The mixture was warmed at 130 °C for 15 h and the solvent was removed in vacuo. The crude product was purified by flash column chromatography on silica gel (10→50% NH_4OH/MeOH) to furnish the final product diamine **2**, in the form of a white solid (4.0 mg, 90% yield); $R_f = 0.31$ in 30% NH_4OH/MeOH; $[\alpha]_D^{22} = -4.6^\circ$ ($c = 0.4$, MeOH); ^1H NMR (500 MHz, D_2O): $\delta = 4.52$ (brs, 1H), 4.25 (d, $J = 11.0$ Hz, 1H), 3.87–3.74 (m, 2H), 3.83 (dd, $J = 9.9$, 9.9 Hz, 1H), 3.56–3.43 (m, 2H), 3.31–3.22 (m, 1H), 2.32–2.26 (m, 1H), 2.12 (ddd, $J = 12.0$, 12.2, 12.3 Hz, 1H), 2.05–1.95 (m, 1H), 1.91–1.82 ppm (m, 1H); ^{13}C NMR (125 MHz, D_2O): $\delta = 78.8$, 69.9, 67.8, 66.4, 65.2, 52.5, 50.4, 31.6, 27.7 ppm; IR (neat): $\tilde{\nu} = 3440$, 3166, 2954, 1645, 1042, 738 cm^{-1}; HRMS-ESI: *m/z*: calcd for $C_{10}H_{20}N_2NaO_5$: 271.1270 $[M+Na]^+$, found: 271.1266.

Upjohn dihydroxylation of minor six-membered spiro analogue 31 and synthesis of *N*,*N*′-((6*R*,7*S*,9*R*,10*S*,11*R*)-3,4,10,11-tetrahydroxy-1-oxaspiro[5.5]undecane-7,9-diyl)diacetamide (41): *N*-Methylmorpholine *N*-oxide (6 mg, 0.05 mmol) and then OsO_4 (2.5 wt% in *t*BuOH, 17 μL, 1.7×10^{-3} mmol, 9 mol%) were added to a stirred solution of the minor olefin (5 mg, 0.02 mmol) in a mixture of acetone/H_2O (1:1 (*v/v*), 2.0 mL), and the solution was allowed to stir at 0 °C for 4 h. The solvent was removed in vacuo, and the crude product was purified by flash column chromatography on silica gel (CH_2Cl_2→50% MeOH/CH_2Cl_2) to furnish the white crystalline mixture of diastereomeric products (2:1 *dr*; 4.6 mg, 82% overall yield). **41** *major product*: $R_f = 0.25$ in 40% MeOH/CH_2Cl_2; ^1H NMR (500 MHz, CD_3OD): $\delta = 4.25$ (m, 1H), 3.93 (m, 1H), 3.85–3.72 (m, 4H), 3.68 (m, 2H), 3.35 (m, 2H), 2.14 (dd, $J = 4.7$, 14.3 Hz, 1H), 1.99 (dt, $J = 4.5$, 12.0 Hz, 1H), 1.94 (s, 3H), 1.93 (s, 3H), 1.82 (dd, $J = 6.9$, 14.3 Hz, 1H), 1.34 ppm (q, $J = 12.0$ Hz, 1H).

Synthesis of final epimeric products (6*R*,7*S*,9*R*,11*S*)-9,11-diamino-1-oxaspiro[5.5]dodecane-3,4,7,8-tetraol (42): Diacetamides **41** (4.0 mg, 0.012 mmol) were dissolved in H_2O (2.0 mL), and KOH(s) (0.12 g, 2.1 mmol) was added. The mixture was warmed at 120 °C for 17 h, and the solvent was removed in vacuo. The crude product was purified by flash column chromatography on silica gel (10→50% NH_4OH/MeOH) to furnish the final diastereomeric diamines **42** (2:1 *dr*), as a white solid. (2.5 mg, 85% yield); **42** *major product*: $R_f = 0.32$ in 30% NH_4OH/MeOH; ^1H NMR (500 MHz, D_2O): $\delta = 4.41$ (m, 1H), 4.27 (d, $J = 12.1$ Hz, 1H), 3.86 (d, $J = 12.1$ Hz, 1H), 3.81 (m, 1H), 3.59–3.51 (m, 2H), 3.22–3.18 (m, 2H), 2.21 (m, 1H), 1.78–1.71 (m, 2H), 1.64 ppm (t, $J = 12.4$ Hz, 1H); ^{13}C NMR (63 MHz, D_2O): $\delta = 81.5$, 78.3, 73.4, 70.0, 68.2, 66.6, 54.8, 52.1, 28.0, 27.0 ppm; HRMS-ESI: *m/z*: calcd for $C_{10}H_{20}N_2NaO_5$: 271.1270 $[M+Na]^+$, found: 271.1265.

Synthesis of *N*,*N*′-((3*S*,4*S*,5*S*,6*S*,8*R*,9*S*,10*R*)-3,4,9,10-tetrahydroxy-1-oxaspiro[4.5]decane-6,8-diyl)diacetamide (44): A solution of **27** (11 mg, 0.039 mmol) and TMEDA (6.9 μL, 0.046 mmol) in CH_2Cl_2 (4.8 mL) was cooled to −78 °C, and OsO_4 (2.5 wt% in *t*BuOH, 0.58 mL, 0.046 mmol) was added. The solution was stirred for 3 h

while slowly warming to RT. The solvent was removed in vacuo, and the crude product was purified by flash column chromatography on silica gel ($CH_2Cl_2 \rightarrow 20\%$ MeOH/CH_2Cl_2) to furnish osmate ester **43** (19 mg, 75% yield) as a yellow solid; $R_f = 0.13$ in 7% MeOH/CH_2Cl_2.

The product was dissolved in MeOH (1.0 mL), and concd HCl (1 drop) was added. The solution was stirred for 15 min until a yellow precipitate was observed. The solvent was removed in vacuo, and the crude product was purified by flash chromatography on silica gel ($20 \rightarrow 50\%$ MeOH/CH_2Cl_2) to furnish diol **44** (9.2 mg, quant.) as a white solid; $R_f = 0.52$ in 50% MeOH/CH_2Cl_2; $[\alpha]_D^{22} = +65.0^\circ$ ($c = 0.2$, MeOH); ^{1}H NMR (500 MHz, CD_3OD): $\delta = 7.93$ (d, $J = 9.2$ Hz, 1H), 4.13–4.08 (m, 3H), 3.96 (m, 1H), 3.88 (d, $J = 10.0$ Hz, 1H), 3.71 (m, 1H), 3.56–3.50 (m, 2H), 1.95 (s, 6H), 1.81 (m, 1H), 1.65 ppm (q, $J = 12.5$ Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD): $\delta = 172.1$, 171.9, 85.9, 74.3, 73.6, 73.3, 72.9, 71.4, 49.8, 48.7, 32.9, 21.3, 21.1 ppm; IR (neat): $\tilde{\nu} = 3363$, 1635, 1559, 1376, 1036, 527 cm^{-1}; HRMS-ESI: *m/z*: calcd for $C_{13}H_{23}N_2O_7$: 319.1500 $[M+H]^+$, found: 319.1498.

Synthesis of final product (3*S*,4*S*,5*S*,6*R*,7*S*,8*R*,10*S*)-8,10-diamino-1-oxaspiro[4.5]decane-3,4,6,7-tetraol (1): Tetraol diacetamide **44** (12 mg, 0.036 mmol) was dissolved in H_2O (2.8 mL) and KOH(s) (0.14 g, 2.5 mmol) was added. The mixture was warmed at 140 °C for 48 h and the solvent was removed in vacuo. The crude product was purified by flash column chromatography on silica gel (MeOH $\rightarrow$ 25% NH_4OH/MeOH) to furnish the final product diamine **1**, as a white solid (1.7 mg, 20% yield); $R_f = 0.46$ in 25% NH_4OH/MeOH; ^{1}H NMR (500 MHz, D_2O): $\delta = 4.45$ (d, $J = 5.5$ Hz, 1H), 4.21 (m, 1H), 3.95–3.90 (m, 2H), 3.58–3.55 (m, 2H), 3.33–3.29 (m, 1H), 3.15 (m, 1H), 2.21 (m, 1H), 1.74 ppm (m, 1H); ^{13}C NMR (125 MHz, D_2O): $\delta = 77.7$, 75.0, 72.4, 71.3, 71.2, 70.4, 50.0, 49.8, 30.2 ppm; IR (neat): $\tilde{\nu} = 3222$, 1627, 1436, 1185, 1048, 864, 585 cm^{-1}; HRMS-ESI: *m/z*: calcd for $C_9H_{19}N_2O_5$: 235.1289 $[M+H]^+$, found: 235.1289.

Synthesis of (5*R*,6*R*,7*S*,8*R*,10*S*)-8,10-diamino-1-oxaspiro[4.5]dec-3-ene-6,7-diol (45): According to the hydrolysis step described for **32**, 5-membered unsaturated analogue **45** was isolated (5 mg, quant.). $R_f = 0.15$ in 30% NH_4OH/MeOH; ^{1}H NMR (500 MHz, D_2O): $\delta = 6.37$ (d, $J = 6.2$ Hz, 1H), 5.75 (m, 1H), 4.71 (m, 2H), 3.71 (m, 1H), 3.48 (d, $J = 10.0$ Hz, 1H), 3.23 (t, $J = 9.8$ Hz, 1H), 2.92 (d, $J = 12.3$ Hz, 1H), 2.81 (m, 1H), 2.01 (m, 1H), 1.38 ppm (q, $J = 12.7$ Hz, 1H); ^{13}C NMR (125 MHz, D_2O): $\delta = 133.4$, 121.3, 97.9, 77.0, 76.7, 76.1, 52.5, 50.9, 35.4 ppm; HRMS-ESI: *m/z*: calcd for $C_9H_{16}N_2NaO_3$: 223.1059 $[M+Na]^+$, found: 223.1057.

Upjohn dihydroxylation of minor seven-membered spiro analogue 24 and synthesis of *N*,*N*′-((2′*S*,3a*R*,5*S*,5′*R*,6′*S*,7*R*,7a*S*)-2,2-cyclohexyl-5′,6′-dihydroxytetrahydro-3a*H*-spiro[benzo[d][1,3]dioxole-4,2′-oxepane]-5,7-diyl)diacetamide (46) and its 5′*S*,6′*R* isomer (47): Spiroalkene **24** (45 mg, 0.11 mmol) was dissolved in a mixture of acetone/H_2O (1:1 (*v/v*), 4.6 mL). *N*-Methylmorpholine *N*-oxide (41 mg, 0.34 mmol) and OsO_4 (0.11 mL, 0.011 mmol, 2.5% (*w/w*)) were added, and the reaction mixture was stirred vigorously at RT. After 13 h, the mixture was concentrated under reduced pressure, and the residue was purified by flash column chromatography on silica gel ($CH_2Cl_2 \rightarrow 10\%$ MeOH/CH_2Cl_2) to afford the corresponding diastereomeric vicinal diols **46**–**47** (36 mg, 70% yield) as a yellowish solid (2:3 mixture of diastereoisomers); $R_f = 0.26$ in 30% MeOH/CH_2Cl_2; ^{1}H NMR (500 MHz, CD_3OD): $\delta = 4.29$ (d, $J = 13.5$ Hz, 1H; major), 4.24 (d, $J = 9.6$ Hz, 1H; minor), 4.00–3.90 (m, 4H), 3.86 (dd, $J = 4.2$, 13.5 Hz, 2H), 3.76 (brs, 1H; minor), 3.73 (brs, 1H; major), 3.67–3.60 (m, 2H), 3.55 (d, $J = 9.6$ Hz, 2H), 3.44 (d, $J = 8.7$ Hz, 1H; minor), 3.40 (d, $J = 8.7$ Hz, 1H; major), 2.15–2.00 (m, 4H), 1.97 (d, $J = 8.0$ Hz, 6H), 1.94 (s, 6H), 1.93–1.85 (m, 2H), 1.84–1.69 (m, 4H), 1.68–1.50 (m, 18H), 1.49–1.31 ppm (m, 4H); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 171.9$, 171.8, 171.7, 171.6, 111.3, 84.0, 83.8, 77.9, 75.5, 75.2, 73.6, 72.7, 71.7, 65.2, 64.7, 54.7, 54.0, 48.0, 36.4, 36.3, 34.5, 34.2, 29.1, 26.8, 26.3, 25.1 (2C), 23.8 (2C), 21.8, 21.7 ppm; HRMS-ESI: *m/z*: calcd for $C_{21}H_{34}N_2NaO_7$: 449.2264 $[M+Na]^+$, found: 449.2263.

Removal of the ketal and synthesis of *N*,*N*′-((1*S*,3*R*,4*S*,5*R*,6*S*,9*S*,10*R*)-4,5,9,10-tetrahydroxy-7-oxaspiro[5.6]dodecane-1,3-diyl)diacetamide (48) and its 9*R*,10*S* isomer (49): The aforementioned diols **46**–**47** (23 mg, 0.054 mmol) were dissolved in a mixture of TFA/THF/H_2O (3:3:1 (*v/v/v*), 0.6 mL) and the mixture was stirred at 50 °C for 18 h. The mixture was then concentrated under reduced pressure, and the residue was purified by flash column chromatography on silica gel ($10\% \rightarrow 30\%$ MeOH/CH_2Cl_2) to afford the corresponding tetraols **48**–**49** (14 mg, 75% yield) as a white solid (2:3 mixture of stereoisomers); $R_f = 0.63$ in 30% MeOH/CH_2Cl_2; ^{1}H NMR (500 MHz, CD_3OD): $\delta = 4.55$ (dd, $J = 4.0$, 13.5 Hz, 1H; major), 4.29 (dd, $J = 8.0$, 12.0 Hz, 1H; minor), 3.85 (dd, $J = 3.5$, 12.0 Hz, 1H), 3.78–3.63 (m, 2H), 3.61–3.53 (m, 2H), 3.35–3.30 (m, 1H), 3.26 (d, $J = 9.5$ Hz, 1H; major), 3.17 (d, $J = 9.5$ Hz, 1H; minor), 2.41–2.19 (m, 1H), 2.16–2.03 (m, 1H), 1.97 (d, $J = 4.5$ Hz, 3H), 1.95 (s, 3H), 1.82–1.70 (m, 2H), 1.70–1.55 (m, 4H), 1.34–1.17 ppm (m, 2H); ^{13}C NMR (125 MHz, CD_3OD): $\delta = 172.3$, 171.3, 79.1, 75.8, 73.7, 73.3, 73.0, 65.2, 54.2, 50.7, 33.0, 28.9, 27.3, 21.8, 21.7 ppm; HRMS-ESI: *m/z*: calcd for $C_{15}H_{26}N_2NaO_7$: 369.1638 $[M+Na]^+$, found: 369.1633.

Synthesis of (1*R*,2*S*,3*R*,5*S*,6*S*,9*R*,10*S*)-3,5-diamino-7-oxaspiro[5.6]dodecane-1,2,9,10-tetraol (3) and its 9*S*,10*R* isomer (50): Tetraols **48**--**49** (6.0 mg, 0.024 mmol) were dissolved in H_2O (2.0 mL), KOH (0.10 mg, 1.7 mmol) was added, and the mixture was stirred for 14 h at 120 °C. Subsequently, the reaction mixture was concentrated under reduced pressure and purified by flash column chromatography on silica gel ($10\% \rightarrow 30\%$ NH_4OH/MeOH) to afford **50** (2.3 mg, 28% yield) and **3** (3.4 mg, 42% yield) as white solids.

50: $R_f = 0.63$ in 30% NH_4OH/MeOH; $[\alpha]_D^{22} = +2.8^\circ$ ($c = 0.2$, MeOH); ^{1}H NMR (D_2O; 500 MHz): $\delta = 4.28$ (d, $J = 13.4$ Hz, 1H), 3.87 (dd, $J = 5.2$, 13.4 Hz, 1H), 3.84 (d, $J = 3.4$ Hz, 1H), 3.82 (d, $J = 5.2$ Hz, 1H), 3.78–3.74 (dd, $J = 3.4$, 11.0 Hz, 1H), 3.51–3.41 (m, 1H), 3.27 (d, $J = 9.6$ Hz, 1H), 2.80–2.73 (m, 1H), 2.31–2.19 (m, 1H), 2.12–2.03 (m, 1H),1.82–1.55 ppm (m, 4H); ^{13}C NMR (D_2O, 125 MHz): $\delta = 79.8$, 78.5, 75.2, 74.6, 73.1, 64.8, 54.2, 51.1, 28.7, 26.4, 22.5 ppm; IR (neat): $\tilde{\nu} = 3120$, 3030, 2837, 1589, 1496, 1438, 1042 cm^{-1}; HRMS-ESI: *m/z*: calcd for $C_{11}H_{22}N_2NaO_5$: 285.1426 $[M+Na]^+$, found: 285.1417.

3: $R_f = 0.31$ in 30% NH_4OH/MeOH; $[\alpha]_D^{22} = -2.6^\circ$ ($c = 0.1$, MeOH); ^{1}H NMR (D_2O; 500 MHz): $\delta = 4.49$ (d, $J = 14.6$ Hz, 1H), 3.97–3.84 (m, 2H), 3.84 (d, $J = 10.2$ Hz, 1H), 3.82–3.74 (m, 1H), 3.48–3.43 (dd, $J = 3.2$, 10.2 Hz, 1H), 3.38 (d, $J = 10.2$ Hz, 1H), 3.34–3.27 (m, 1H), 2.52 (t, $J = 13.4$ Hz, 1H), 2.30–2.21 (m, 1H), 1.94–1.68 (m, 4H); ^{13}C NMR (D_2O, 125 MHz): $\delta = 78.2$, 77.1, 75.1, 72.2, 70.8, 65.5, 55.9, 51.1, 28.7, 27.7, 27.3; IR (neat): $\tilde{\nu} = 3118$, 3028, 1641, 1442, 1392, 1013 cm^{-1}; HRMS-ESI: *m/z*: calcd for $C_{11}H_{22}N_2NaO_5$: 285.1426 $[M+Na]^+$, found: 285.1419.

Bacterial decoding site construct: To investigate specific conformational events in solution, an RNA construct with 2-aminopurine (2AP) instead of adenine 1492 was utilized. The construct comprises the bacterial A-site sequence and flanking auxiliary base pairs designed to form a double helix (Scheme 1). Gel-purified and desalted oligonucleotides were purchased from Dharmacon Inc. (Lafayette, CO, USA). Double-stranded constructs were obtained by annealing complementary strands in sodium cacodylate buffer (30 mM, pH 6.8) at 65 °C for 3 min and snap cooling on ice.

Fluorescence binding assay: Titrations of single-stranded oligonucleotide **A** to **B** bearing the 2AP label was performed at ratios ranging from 0.125:1 to 2:1 (**A** to **B**), at which emission spectra were recorded at 1 μM concentration (30 mM sodium cacodylate, pH 6.8) in 1 cm path-length quartz cells. Hybridization was confirmed with analytical gel electrophoresis.

Titrations of tested compounds to the 2AP-labeled RNA bipartite construct were performed, with concentrations ranging from 1 mM to 1 nM. Emission spectra were recorded at 1 μM RNA concentration (30 mM sodium cacodylate, pH 6.8) in 1 cm path-length quartz cells.

Fluorescence was measured on a Cary Eclipse (Varian) fluorescence spectrophotometer at 25 °C. The excitation wavelength used was at 310 nm, and emission was monitored between 320 and 450 nm and normalized at 370 nm. Half-maximal response concentration (EC_{50}) values were calculated by fitting a dose–response curve to the fluorescence intensity plotted against the log of the ligand concentration. Three replicate experiments were run per compound. All scaffolds under investigation were also added to the 2AP-labeled single-stranded **B** oligonucleotide as a control experiment. Titrations with spermidine, a nonspecific RNA binder, were performed as negative dose–response controls.

Bacterial in vitro transcription translation: The coupled in vitro transcription–translation assay was carried out in a 96-well format. The tested compounds were incubated at different concentrations, ranging from 1 mM to 500 nM, with pBEST*luc* (Promega) plasmid DNA containing the gene encoding luciferase, under transcriptional control of P*tac* promoter. Plasmid DNA was incubated at a concentration of 200 ng μL^{-1}, in a mixture of bacterial extract, premix, and amino acids, in a total reaction volume of 10 μL at 37 °C for 30 min. After cooling on ice for 5 min, luciferin substrate (Promega) was added and luminescence was measured immediately with an Infinite M200 (Tecan) luminescence counter. Half-maximal inhibitory concentration (IC_{50}) values were determined from luminescence intensity plotted versus the log of compound concentrations by fitting to a variable slope dose–response equation. Three replicate experiments were run per compound. Positive and negative control experiments were also performed for each compound.

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