# Backbone NMR assignments of the anti-CRISPR AcrIIA5 from phages infecting Streptococcus thermophilus 

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Received Sep 10, 2020; Revised Sep 17, 2020; Accepted Sep 17, 2020


#### Abstract

The CRISPR-Cas system provides an adaptive immunity for bacteria and archaea against invading phages or foreign plasmids. In the type II CRISPR-Cas system, a single effector protein Cas9 and a guide RNA form an RNA-guided endonuclease complex that can degrade DNA targets of foreign origin. To avoid the Cas9-mediated destruction, phages evolved anti-CRISPR (Acr) proteins that neutralize the host bacterial immunity by inactivating the CRISPR-Cas system. Here we report the backbone ${ }^{1} \mathrm{H},{ }^{15} \mathrm{~N}$, and ${ }^{13} \mathrm{C}$ resonance assignments of AcrIIA5 that inhibits the endonuclease activity of type II-A Streptococcus thermophilus Cas9 and also Streptococcus pyogenesis Cas9 using triple resonance nuclear magnetic resonance spectroscopy. The backbone chemical shifts of AcrIIA5 predict a disordered region at the N -terminus, followed by an $\alpha \beta \beta \beta \beta \alpha \beta \beta \beta$ fold.


Keywords AcrIIA5, anti-CRISPR, Cas9, NMR spectroscopy

## Introduction

The clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas)
proteins constitute an adaptive immunity of bacteria and archaea against bacteriophages and foreign plasmids ${ }^{1,2}$. Upon infection, bacteria activates the CRISPR-Cas system to integrate short segments (spacers) of invading nucleic acids into the CRISPR loci of the host genome. Upon subsequent invasion, CRISPR RNA (crRNA) and sometimes transactivating CRISPR RNA (tracrRNA) as well are transcribed and processed to associate with Cas proteins, and the RNA-guided endonuclease complex recognizes and destroys nucleic acids that are complementary to the guide sequence of crRNA. The CRISPR-Cas systems are divided into two classes based on the nature of the interference complex (multiple subunits or a single effector), and further into various types and subtypes according to the phylogenic and genetic signature ${ }^{3}$. Streptococcus pyogenes Cas9 (SpyCas9) belongs to the subtype II-A of the Class2 CRISPR-Cas system. SpyCas9 has been extensively investigated and applied to genome editing due to its simplicity in targeting double-strand DNA at desired sites guided by single-guide RNA (sgRNA) ${ }^{4-6}$.
Arms-race between bacteria and phages have long been evolved, developing a variety of arsenal to compete for invasion and defense ${ }^{7}$. Recently AcrIIA5 encoded by virulent phages infecting Streptococcus

[^0]thermophilus prophages have shown a wide inhibitory activity against different Cas9 proteins including SpyCas9, highlighting their potential as an off-switch for Cas9 to control gene editing ${ }^{8}$. Biochemical and cell-based assays have been employed to reveal that AcrIIA5 inhibits a broad range of Cas9 orthologs, preventing the cleavage of target $\mathrm{DNA}^{9,10}$. Here we report the backbone chemical shifts and secondary structures of AcrIIA5 using triple-resonance NMR spectroscopy.

## Experimental Methods

Sample preparation- The synthetic AcrIIA5 (a.a. 1140) gene was cloned into a pET28 vector with an N terminal $\mathrm{His}_{6}$-tag and a maltose binding protein (MBP) tag, and verified by DNA sequencing. The plasmid was introduced into Escherichia coli strain BL21star(DE3) cells (Invitrogen), and grown minimal media with ${ }^{15} \mathrm{NH}_{4} \mathrm{Cl}$ and/or ${ }^{13} \mathrm{C}_{6}$-glucose as sole nitrogen or carbon sources, respectively. Cells were grown at $37^{\circ} \mathrm{C}$ to $\mathrm{OD}_{600} 0.6 \sim 0.8$, induced with 1 mM isopropyl- $\beta$-D-thiogalactopyranoside (IPTG) at $18^{\circ} \mathrm{C}$, and harvested by centrifugation after 20 hours of induction. Cell pellets were resuspended in 20 mM Tris-HCl, pH 7.4, $500 \mathrm{mM} \mathrm{NaCl}, 5 \%$ glycerol, 5 mM $\beta$-mercaptoethanol (buffer A) with $1 \quad \mathrm{mM}$ phenylmethylsulfonyl-fluoride(PMSF), lysed using Emulsiflex C3 (AVESTIN) and centrifuged at 40,000 g for 30 min . The clear supernatant was loaded onto a HisTrap column (GE Healthcare) equilibrated with buffer A and eluted with 500 mM imidazole. The $\mathrm{His}_{6}$ tag and maltose binding protein (MBP) was removed using TEV protease in buffer A with 0.5 mM EDTA, and the digestion reaction was loaded onto the SP column(GE Healthcare) and eluted with a $0-1 \mathrm{M} \mathrm{NaCl}$ gradient. The flow-through was collected and loaded onto a Superdex 75 column (GE Healthcare) equilibrated with buffer A.

NMR experiments - The NMR sample contained 0.6 $\mathrm{mM}{ }^{13} \mathrm{C},{ }^{15} \mathrm{~N}$-AcrIIA5 in with 10 mM sodium phosphate, $\mathrm{pH} 7.0,500 \mathrm{mM} \mathrm{NaCl}, 5 \mathrm{mM} \beta-$ mercaptoethanol and $10 \% \mathrm{D}_{2} \mathrm{O}$. NMR spectra were
recorded at $25^{\circ} \mathrm{C}$ on 900 MHz spectrometers equipped with a z-shielded gradient triple resonance probe. Sequential backbone assignments of ${ }^{1} \mathrm{H},{ }^{15} \mathrm{~N}$ and ${ }^{13} \mathrm{C}$ resonances were achieved by three-dimensional triple resonance through-bond scalar correlation experiments $\mathrm{CBCA}(\mathrm{CO}) \mathrm{NH}, \mathrm{HNCACB}, \mathrm{HNCO}$, HN(CA)CO, NMR spectra were processed using the NMRPipe ${ }^{11}$, and analyzed using PIPP $^{12}$, and NMRView ${ }^{13}$ programs. Overall secondary structure and the backbone dihedral torsion angles were predicted from TALOS+ based on combined $\mathrm{H}_{\mathrm{N}},{ }^{15} \mathrm{~N}$, $\mathrm{C} \alpha, \mathrm{C} \beta$ and $\mathrm{C}^{\prime}$ backbone chemical shifts ${ }^{14}$.

## Results and Discussion

Backbone assignment of AcrIIA5- AcrIIA5 has 140 amino acids and exhibited a well dispersed $2 \mathrm{D}{ }^{1} \mathrm{H}^{15} \mathrm{~N}$ HSQC NMR spectrum (Figure 1A). We noticed that the HSQC spectra of ${ }^{15} \mathrm{~N}$-labeled AcrIIA5 in 20 mM Tris, pH 7.4, showed fewer backbone amide resonances than expected. When we changed the buffer to 10 mM sodium phosphate, pH 7.0 , a dozen of new resonances appeared (Figure 1A). Thus, we used pH 7.0 for the sequential backbone assignment of AcrIIA5, and obtained a suite of three-dimensional heteronuclear correlation NMR spectroscopy, such as $\mathrm{HNCO}, \mathrm{HN}(\mathrm{CA}) \mathrm{CO}, \mathrm{CBCA}(\mathrm{CO}) \mathrm{NH}$, and HNCACB, using a standard protocol ${ }^{15,16}$. The backbone chemical shifts for 118 out of 140 residues were assigned, and the residues without assignments did not appear in the 2D ${ }^{1} \mathrm{H}^{-15} \mathrm{~N}$ HSQC spectra, suggesting a line broadening by conformational exchanges.
Amino-acid selective isotope labeling is often useful to resolve ambiguous assignments. It has also been reported that reverse isotope labeling can help assignments by selectively filtering out resonances of particular amino acids. We tried the reverse isotope labeling of Leu to help the assignment of AcrIIA5. Briefly, ${ }^{15} \mathrm{NH}_{4} \mathrm{Cl}$ was used in the same manner as the uniform 15 N -labeling protocol, and in addition, 50 mg of unlabeled leucine was introduced before induction to dilute the isotope-labeling of leucine positions. ${ }^{17}$ The reverse labeling of Leu indeed suppressed the peak intensity of Leu resonances (Figure 1B).

However, resonances of other aliphatic amino acids also appeared with reduced intensity, possibly from scrambling of biosynthetic pathways. For example, Ile117 and Ala119, among others, exhibited lower intensity compared to those from uniformly ${ }^{15} \mathrm{~N}$ labeled AcrIIA5 (Figure 1B). Nevertheless, clarification of Leu residues with a long spin system facilitated fast and reliable backbone assignment. The ${ }^{1} \mathrm{H},{ }^{15} \mathrm{~N}$ and ${ }^{13} \mathrm{C}$ chemical shifts from the backbone assignment of AcrIIA5 are listed in Table 1, and the annotated HSQC spectrum is shown in Figure 2.


Figure 1. (A) The superimposed ${ }^{1} \mathrm{H}^{-15} \mathrm{~N}$ HSQC spectra of ${ }^{15} \mathrm{~N}$-AcrIIA5 in pH 7.4 (black) and pH 7.0 (red). (B) The superimposed ${ }^{1} \mathrm{H}-{ }^{15} \mathrm{~N}$ HSQC spectra of uniformly ${ }^{15} \mathrm{~N}$ labeled AcrIIA5 (black), and Leu-reverse-labeled AcrIIA5 (red).


Figure 2. ${ }^{1} \mathrm{H}-{ }^{15} \mathrm{~N}$ HSQC spectra of ${ }^{15} \mathrm{~N}$-AcrIIA5 with backbone chemical shift assignments obtained by tripleresonance heteronuclear NMR experiments.

Disorder prediction of AcrIIA5- We noticed that the several backbone amide resonances in the HSQC spectrum appeared in the narrow range between 8 ppm and 8.5 ppm of the ${ }^{1} \mathrm{H}$ chemical shifts, which is typical of a disordered region. We examined if the amino acid sequence of AcrIIA5 would have the characteristic of intrinsically disordered region. We employed two prediction programs, PONDR and IUPred2, to assess the disorder score of AcrIIA5 (Figure 3) ${ }^{18,19}$.
Interestingly, both programs predicted that the N terminal region of AcrIIA5 would behave as a disordered region. The low complexity of the amino acid sequence and the presence of disorder-promoting motifs suggested that $20-30$ residues at the N terminus potentially remain disordered without welldefined secondary structures, albeit residues at the far end of the N -terminus exhibited less disorder probability than those near the inner core of the protein

Secondary structure annotation and order parameters of AcrIIA5- Secondary structural information and predicted order parameters of AcrIIA5 were calculated using TALOS+ program based on the ${ }^{1} \mathrm{H}_{\mathrm{N}},{ }^{15} \mathrm{~N},{ }^{13} \mathrm{C} \alpha$, ${ }^{13} \mathrm{C} \beta$, and ${ }^{13} \mathrm{C}^{\prime}$ chemical shifts (Figure 4 A ) ${ }^{14}$. The secondary structure prediction indicated that AcrIIA5 is comprised of two $\alpha$-helices, seven $\beta$-strands, and a $3_{10}$ helix. The structure of AcrIIA5 determined by NMR spectroscopy was largely consistent with the
secondary structures predicted by the backbone torsion angles from the chemical shifts ${ }^{20}$. We note that the predicted order parameters from the random coil index suggest a significant flexibility at the N -terminal disordered region and the $\beta 3-\beta 4$ loop region (Figure 4B).
In summary, we report the optimal buffer condition and backbone chemical shift assignment of AcrIIA5. Both the amino acid sequence and NMR chemical shifts predict a long stretch of flexible disordered region at the N -terminus of AcrIIA5.


Figure 3. Disorder scores from the sequence-based prediction programs of (A) PONDR, and (B) IUPred2. The predicted disorder regions are highlighted in yellow shades and annotated.


Figure 4. (A) Predicted secondary structures and (B) order parameters of AcrIIA5 based on backbone chemical shifts. Blue and red bars denote the predicted region of $\beta$-sheets and $\alpha$-helices, respectively. The schematic representations of secondary structures are displayed at the top of (B). Order parameters were calculated via the random coil index using backbone chemical shifts.

Table 1. Backbone $\mathrm{H}_{\mathrm{N}}, \mathrm{N}, \mathrm{C} \alpha, \mathrm{C} \beta$, and $\mathrm{C}^{\prime}$ chemical shifts of AcrIIA5 (unit: ppm)

| Residue | $\mathrm{H}_{\mathrm{N}}$ | N | $\mathrm{C} \alpha$ | C $\beta$ | $\mathrm{C}^{\prime}$ | Residue | $\mathrm{H}_{\mathrm{N}}$ | N | $\mathrm{C} \alpha$ | C $\beta$ | $\mathrm{C}^{\prime}$ |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| M1 | 8.438 | 122.56 | 55.70 | 32.94 | 175.45 | K71 | 7.789 | 119.11 | 56.40 | 33.35 | 175.56 |
| A2 | 8.350 | 125.73 | 52.37 | 19.42 | 177.14 | Y72 | 7.910 | 119.42 | 58.17 | 38.30 | 175.44 |
| Y3 | 8.156 | 119.58 | 58.05 | 38.85 | 176.45 | H73 | 7.320 | 121.55 | 56.39 |  | 173.33 |
| G4 | 8.336 | 110.45 | 45.47 |  | 174.15 | D74 |  |  |  |  |  |
| K5 | 8.146 | 120.93 | 56.55 | 33.13 | 176.83 | L75 |  |  |  |  |  |
| S6 | 8.330 | 116.45 | 58.79 | 63.94 | 174.61 | E76 |  |  |  |  |  |
| R7 | 8.287 | 122.51 | 56.52 | 30.75 | 176.07 | N77 |  |  | 53.92 | 38.29 | 175.66 |
| Y8 | 8.113 | 120.14 | 58.02 | 38.76 | 175.62 | G78 | 8.454 | 105.45 | 45.57 |  | 173.60 |
| N9 | 8.245 | 120.32 | 53.42 | 39.02 | 175.76 | R79 | 7.866 | 120.75 | 54.92 | 31.93 | 174.70 |
| S10 |  |  |  |  |  | L80 | 8.423 | 126.46 | 55.53 | 42.30 | 173.91 |
| Y11 |  |  |  |  |  | I81 |  |  | 58.97 | 37.93 | 174.12 |
| R12 |  |  |  |  |  | V82 | 8.706 | 126.34 | 60.22 | 32.06 | 174.13 |
| K13 | 8.314 | 120.05 | 56.73 | 32.75 | 177.67 | N83 | 8.935 | 126.47 | 53.15 | 43.08 | 173.38 |
| R14 | 8.299 | 122.06 | 56.52 | 30.94 | 176.33 | 184 | 9.457 | 126.90 | 60.01 | 39.77 | 174.67 |
| S15 | 8.227 | 116.67 | 58.40 | 63.99 | 174.21 | K85 | 7.879 | 127.12 | 56.48 | 33.19 | 176.36 |
| F16 | 8.244 | 122.17 | 57.98 | 39.78 | 175.34 | A86 | 8.420 | 130.29 | 52.10 | 22.92 | 174.67 |
| N17 | 8.381 | 120.46 | 53.42 | 39.22 | 175.25 | S87 | 8.337 | 113.49 | 56.69 | 64.64 | 175.98 |
| R18 |  |  | 56.61 | 30.76 | 176.46 | K88 | 8.486 | 124.32 | 57.99 | 32.00 | 177.30 |
| S19 | 8.374 | 116.48 | 58.77 | 64.06 | 174.55 | L89 | 7.608 | 116.98 | 56.43 | 41.16 | 177.35 |
| N20 | 8.485 | 120.98 | 54.00 | 38.95 | 175.67 | N90 | 8.108 | 114.66 | 52.80 | 40.58 | 175.42 |
| K21 | 8.267 | 121.11 | 57.65 | 32.89 | 178.12 | F91 | 7.401 | 118.43 | 58.76 | 38.44 | 176.73 |
| Q22 |  |  |  |  |  | V92 | 8.409 | 117.14 | 67.64 | 31.01 | 176.77 |
| R23 |  |  | 57.06 | 30.55 | 177.24 | D93 | 8.065 | 119.01 | 57.65 | 42.33 |  |
| R24 | 8.382 | 122.26 | 57.56 | 30.67 | 177.14 | 194 | 8.200 | 117.55 | 65.38 | 37.81 | 178.27 |
| E25 | 8.511 | 121.08 | 57.72 | 29.95 | 177.27 | 195 | 8.137 | 121.40 | 66.29 | 38.19 | 178.20 |
| Y26 | 8.213 | 121.35 | 59.70 | 38.64 | 176.47 | E96 | 8.664 | 115.79 | 59.69 | 30.69 | 178.70 |
| A27 | 8.232 | 123.08 | 54.36 | 18.74 | 179.28 | N97 | 8.288 | 111.41 | 54.86 | 42.22 | 176.44 |
| Q28 | 8.170 | 118.18 | 57.78 | 29.00 | 177.82 | K98 | 8.293 | 117.75 | 56.05 | 35.25 | 177.01 |
| E29 | 8.340 | 121.19 | 58.46 | 29.42 | 178.65 | L99 | 8.255 | 121.28 | 57.65 | 42.85 | 178.17 |
| M30 |  |  | 56.91 | 32.44 | 177.65 | D100 | 8.639 | 118.66 | 57.91 | 39.64 | 178.63 |
| D31 | 8.212 | 121.62 | 56.51 | 41.37 | 177.77 | K101 | 7.731 | 117.43 | 58.23 | 32.55 | 178.70 |
| R32 | 8.065 | 119.40 | 59.16 | 30.36 | 178.85 | I102 | 7.280 | 119.03 | 65.49 | 38.75 | 177.14 |
| L33 | 8.066 | 122.75 | 58.26 | 42.45 | 178.19 | I103 |  |  | 65.22 | 37.69 | 176.77 |
| E34 | 8.373 | 118.34 | 60.32 | 29.78 | 179.93 | E104 | 7.847 | 116.64 | 59.06 | 29.74 | 178.68 |
| K35 | 8.199 | 118.91 | 58.91 | 32.13 | 179.36 | K105 | 6.860 | 114.90 | 59.56 | 33.24 | 179.10 |
| A36 | 8.176 | 122.34 | 54.94 | 18.62 | 181.09 | I106 | 7.823 | 117.78 | 64.05 | 37.39 | 177.80 |

Table 1. Backbone $\mathrm{H}_{\mathrm{N}}, \mathrm{N}, \mathrm{C} \alpha, \mathrm{C} \beta$, and $\mathrm{C}^{\prime}$ chemical shifts of AcrIIA5 (continued)

| Residue | $\mathrm{H}_{\mathrm{N}}$ | N | $\mathrm{C} \alpha$ | $\mathrm{C} \beta$ | $\mathrm{C}^{\prime}$ | Residue | $\mathrm{H}_{\mathrm{N}}$ | N | $\mathrm{C} \alpha$ | $\mathrm{C} \beta$ | $\mathrm{C}^{\prime}$ |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| F37 | 8.226 | 116.68 | 61.74 | 39.21 | 178.22 | D107 | 8.466 | 118.08 | 56.31 | 40.16 | 177.80 |
| E38 | 7.843 | 120.66 | 59.06 | 29.58 | 177.39 | K108 | 7.054 | 116.39 | 56.43 | 33.14 | 177.43 |
| N39 | 7.389 | 113.86 | 53.15 | 39.91 | 174.28 | L109 | 7.607 | 119.51 | 54.65 | 43.51 | 177.02 |
| L40 | 7.266 | 121.93 | 53.41 | 41.98 | 175.72 | D110 | 8.561 | 119.96 | 52.78 | 39.10 | 176.34 |
| D41 | 8.495 | 124.31 | 56.10 | 41.46 | 176.94 | L111 | 7.883 | 122.52 | 58.25 | 41.56 | 178.33 |
| G42 | 8.641 | 114.39 | 45.72 |  | 171.72 | D112 | 8.101 | 115.91 | 56.31 | 41.20 | 177.00 |
| W43 | 7.166 | 118.06 | 56.64 | 30.98 | 175.94 | K113 | 7.652 | 114.64 | 57.45 | 32.96 | 178.07 |
| Y44 | 8.949 | 120.32 | 56.79 | 41.32 | 174.49 | Y114 | 7.483 | 116.72 | 58.05 | 40.71 | 173.57 |
| L45 | 8.595 | 122.05 | 53.45 | 44.71 | 177.94 | R115 | 9.034 | 114.28 | 54.70 | 31.47 | 174.95 |
| S46 | 8.802 | 118.59 | 58.04 | 64.71 | 176.97 | F116 | 7.403 | 120.62 | 58.56 | 41.93 | 172.52 |
| S47 |  |  |  |  |  | I117 | 7.939 | 129.27 | 60.39 | 40.94 | 171.69 |
| M48 |  |  |  |  |  | N118 | 9.348 | 124.03 | 51.11 | 42.15 |  |
| K49 |  |  |  |  |  | A119 | 9.787 | 133.60 | 50.99 | 18.91 | 176.60 |
| D50 |  |  | 53.16 | 41.20 | 175.57 | T120 | 8.460 | 117.55 | 63.37 | 69.39 | 173.36 |
| S51 | 7.999 | 113.01 | 57.52 | 66.26 | 171.53 | N121 | 9.950 | 126.76 | 52.00 | 38.33 | 173.44 |
| A52 | 8.655 | 123.53 | 50.50 | 24.17 | 176.25 | L122 | 9.254 | 118.93 | 56.86 | 39.30 | 175.50 |
| Y53 | 9.212 | 118.14 | 56.44 | 43.01 | 172.69 | E123 |  |  | 57.49 | 28.23 | 174.18 |
| K54 | 8.222 | 118.37 | 56.02 | 30.88 | 174.23 | H124 | 8.016 | 121.25 | 55.58 | 30.43 | 174.28 |
| D55 | 8.860 | 125.82 | 54.24 | 41.67 | 175.97 | D125 | 9.200 | 127.04 | 55.12 | 38.71 | 175.67 |
| F56 | 8.931 | 126.50 | 58.76 | 39.91 | 176.60 | I126 | 8.630 | 122.94 | 63.69 | 38.39 | 175.67 |
| G57 | 9.177 | 114.86 | 46.95 |  | 175.50 | K127 | 8.015 | 125.63 | 56.25 | 34.51 | 174.71 |
| K58 | 7.850 | 120.62 | 57.32 | 32.90 | 175.34 | C128 | 8.766 | 122.32 | 56.37 | 27.92 | 174.00 |
| Y59 | 6.900 | 110.78 | 54.82 | 39.82 | 173.39 | Y129 | 9.001 | 122.41 | 59.17 | 38.41 | 177.01 |
| E60 | 9.014 | 121.42 | 54.82 | 33.76 | 175.46 | Y130 | 8.364 | 124.87 | 60.02 | 40.02 | 177.87 |
| I61 | 8.862 | 125.22 | 60.68 | 38.42 | 174.51 | K131 | 8.690 | 122.31 | 58.21 | 33.92 | 177.21 |
| R62 | 9.159 | 126.33 | 54.44 | 33.98 | 174.45 | G132 | 9.241 | 110.76 | 45.45 |  | 172.83 |
| L63 | 9.403 | 125.16 | 53.65 | 43.21 | 176.51 | F133 | 7.561 | 115.85 | 56.69 | 44.19 | 174.80 |
| S64 | 8.574 | 113.85 | 58.17 | 65.71 | 174.51 | K134 |  |  |  |  |  |
| N65 | 9.292 | 124.20 | 52.34 | 39.50 | 176.10 | T135 |  |  | 61.06 | 70.65 | 174.67 |
| H66 |  |  |  |  |  | K136 | 7.159 | 123.99 | 57.76 | 34.12 | 176.52 |
| S67 | 8.140 | 116.06 | 58.90 | 63.76 |  | K137 | 9.168 | 126.73 | 54.70 | 35.59 | 174.03 |
| A68 | 8.642 | 127.45 | 52.31 | 19.63 | 177.13 | E138 | 8.657 | 119.91 | 54.57 | 33.00 | 174.34 |
| D69 | 8.326 | 119.80 | 54.69 | 41.06 | 176.14 | V139 | 8.600 | 124.93 | 61.68 | 32.89 | 175.88 |
| N70 | 8.248 | 118.04 | 54.09 | 38.52 | 175.15 | I140 | 8.710 | 128.98 | 61.78 | 40.63 | 180.43 |

Backbone NMR assignments of the anti-CRISPR AcrIIA5

## Acknowledgments

This work was supported by the Cooperative Research Program for Agriculture Science \& Technology Development, Rural Development Administration (PJ01495901), and the Creative-Pioneering Researchers Program through Seoul National University (500-20200255). We thank the high-field NMR facility at the Korea Basic Science Institute, and the National Center for Inter-University Research Facilities of Seoul National University.

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