SI Appendix

D-Sedoheptulose-7-phosphate is a common precursor for the heptoses of septacidin and hygromycin B

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Materials and Methods

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table S9.

DNA manipulation and sequence analysis. General DNA manipulations were performed as described (1). PCRs were performed with PrimeSTAR HS DNA polymerase (Takara, Shiga, Japan) or Taq DNA polymerase (TransGene, Beijing, China) according to the manufacturers' instructions. All PCR primers used in this study are listed in Table S10. The λ RED-mediated recombination was performed as described (2). Isolation of *Streptomyces* genomic DNA was performed according to the standard procedure (3). Transformation of *Streptomyces* and *E. coli-Streptomyces* conjugations were carried out according to the standard protocols (4). DNA sequencing of *S. fimriatus* CGMCC 4.1598 was performed with Illumina Hiseq 2000 platform in Majorbio Co. (Shanghai, China). BLAST+ was downloaded (ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/LATEST/) and used for a local searching of genes encoding specific proteins (e.g. homologs of GmhA (NP_414757) and HldE (NP_417524)) on the draft genome of *S. fimriatus* CGMCC 4.1598. The open reading frames (ORFs) were predicted with Prodigal online server (http://prodigal.ornl.gov/). A BLASTP search was used to predict protein functions (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple alignments were performed with CLUSTALW (https://www.ebi.ac.uk/Tools/msa/clustalw2/).

Construction of the *sep* gene cluster heterologous expression strain *S. albus* 1598. Cas9-Assisted Targeting of CHromosome segments (CATCH) was used to capture the 24.0-kb fragment containing the *sep* gene cluster from *S. fimbriatus* CGMCC 4.1598 (5). Briefly, two sgRNA sequences were selected for both regions flanking the *sep* gene cluster. The sep-sgRNAF and sep-sgRNAR *in vitro* transcription templates were prepared by overlapping PCR of 3 primers: a primer (sep-gF-P or sep-gR-P) containing the T7 promoter and target sequence, and 2 others (guide RNA-F and guide RNA-R) carrying crRNA-tracrRNA chimera sequence. Cas9 digestion of *S. fimbriatus* CGMCC 4.1598 genomic DNA plug using sep-sgRNAF and sep-sgRNAR was carried out according to the literature procedures (5). The digested DNA was precipitated with ethanol and suspended in 20 μL DNase-free water. The backbone of the pSET153 vector was amplified from plasmid pSET153 using primers sep-p15A-F and sep-p15A-R, which include a ~30 bp overlap with one end of the target fragment. Fifty nanogram backbone and 1 μg digested genome fragment were assembled using Gibson Assembly Master Mix (NEB). After ligation, the product was transformed into *E. coli* EPI300 competent cells. Plasmid pSET153 containing the *sep* cluster, named pSET1598, was verified by restriction enzyme digestions and transformed into *S. albus* J1074 by intergeneric conjugation to generate the *sep* gene cluster heterologous expression strain *S. albus* 1598.

Construction of the *sep* gene in-frame deletion mutants. All the *sep* gene mutants were constructed by in-frame

gene deletion via λ RED-mediated PCR-targeting method. Briefly, the targeted ORF was replaced with kanamycin resistant gene *aph(3')*-II (*Kan*^{r}) flanking with two isocaudomer cutting sites (*Spe*I and *Bln*I). Then, the recombinant plasmid was double-digested with the two isocaudomers (*Spe*I and *Bln*I) to remove the *Kan*^{r} cassette and self-ligated to generate the in-frame deletion mutant (Fig. S4).

To construct the Δ*sepR* in-frame deletion mutant, a 1.4-kb *Kan*^{r} cassette was amplified using primer pair SepRU/SepRD with plasmid pUK-T5promoter as a template. The 1.4-kb Kan^r cassette was then used to replace the *sepR* gene on pSET1598 via *λ* RED-mediated recombination. The recombinant plasmid was digested with *Spe*I/*Bln*I to remove the Kan^r cassette and self-ligated to form the desired plasmid pSET1598-*ΔsepR*. A similar strategy was adopted in the construction of the plasmids pSET1598-*ΔsepB* (replacing the *sepB* gene on pSET1598 by *Kan*^{r} cassette amplified using primers SepBU/SepBD), pSET1598-*ΔsepC* (replacing the *sepC* gene on pSET1598 by *Kan*^{r} cassette amplified using primers SepCU/SepCD), pSET1598-*ΔsepD* (replacing the *sepD* gene on pSET1598 by *Kan*^{r} cassette amplified using primers SepDU/SepDD), pSET1598-*ΔsepH* (replacing the *sepH* gene on pSET1598 by *Kan*^{r} cassette amplified using primers SepHU/SepHD), pSET1598-*ΔsepL* (replacing the *sepL* gene on pSET1598 by *Kan*^{r} cassette amplified using primers SepLU/SepLD), pSET1598-*ΔsepMNOPQ* (replacing the *sepM*, *sepN*, *sepO*, *sepP*, and *sepQ* genes on pSET1598 together by *Kan*^{r} cassette amplified using primers SepMQU/SepMQD). All the seven pSET1598-derived plasmids were then verified by sequencing. Introduction of the seven plasmids, pSET1598-*ΔsepR*, pSET1598-*ΔsepB*, pSET1598-*ΔsepC*, pSET1598-*ΔsepD*, pSET1598-*ΔsepH*, pSET1598-*ΔsepL* and pSET1598-*ΔsepMNOPQ*, into *S. albus* J1074 by *E. coli-Streptomyces* conjugation generated the mutant stains *S. albus ΔsepR*, *S. albus ΔsepB*, *S. albus ΔsepC*, *S. albus ΔsepD*, *S. albus ΔsepH*, *S. albus ΔsepL*, and *S. albus ΔsepMNOPQ*, respectively.

Production and detection of septacidin and its analogs. The *sep* gene cluster heterologous expression strain *S. albus* 1598 was grown on MS agar with 50 μg/mL apramycin for sporulation. The collected spore suspension was inoculated into seed medium (1% yeast extract, 1% tryptone, 1% (v/v) $CoCl_2$•$6H_2O$ solution (0.05%), 0.25% $CaCO_3$, pH 7.3) with 25 μg/mL apramycin and cultured for 2 days at 28°C on a rotary shaker (220 rpm). Two milliliter seed medium was transferred to 50 mL fermentation medium (1.2% potato starch, 0.8% soybean meal, 0.3% yeast extract, 1% (v/v) $CoCl_2$•$6H_2O$ solution (0.05%), 0.25% $CaCO_3$, pH 7.3) and cultured for another 5 days for production of septacidins. The negative control strain *S. albus* 153 and all the *sep* gene mutant strains were cultured the same as that for production of septacidin and its analogs.

For detection of septacidin and its analogs, the cell pellets were collected by centrifugation and soaked in equal volume of methanol overnight. The methanol extracts were analyzed directly with a ZORBAX CN column (5 μm,

4.6 × 250 mm, Agilent Technologies, Santa Clara, CA, USA) on a Shimadzu HPLC system (Shimadzu, Kyoto, Japan). The column was developed with solvent A (ddH_2O with 0.1% (v/v) trifluoroacetic acid) and acetonitrile at a flow rate of 1.0 mL/min. The percentage of acetonitrile was changed using a linear gradient from 30% to 45% over 0-5 min, and from 45% to 53% over 5-21min. The detection wavelength was 254 nm.

LC-MS was used for analysis of the intermediates accumulated in *S. albus ⊿sepD* and Δ*sepH*. The supernatants of the two mutants were filtrated with a 0.22 μm membrane and analyzed with a ZORBAX SB-AQ column (5 μm, 4.6 × 250 mm, Agilent Technologies, Santa Clara, CA, USA). The column was developed with solvent A (ddH_2O with 0.1% (v/v) formic acid) and acetonitrile at a flow rate of 1.0 mL/min. The percentage of acetonitrile stayed at 1% over 0-3 min and change from 1% to 40% over 3-30 min.

Isolation of SEP-608. The cell pellet of 20 L culture broth of *S. albus*1598 was collected by centrifugation and extracted three times with isometric methanol. The methanol extracts were concentrated *in vacuum* at 35 ^{o}C and subjected to a Sephadex LH-20 column (35 mm × 700 mm) using methanol as the mobile phase. The methanol eluted fractions containing septacidin analogs were concentrated and lyophilized to obtain the crude products. At last, one of the crude products was refined by semi-preparative HPLC (ZORBAX C18, 5 μm, 9.4 mm × 250 mm, Agilent, Santa Clara, CA, USA) eluted with acetonitrile/water (35/65, v/v) containing 0.1% trifluoroacetic acid at a flow rate of 3.7 mL/min to afford 27 mg SEP-608.

Preparation of SEP-327. SEP-327 was prepared using a method modified from literature. Briefly, the methanolysis of 15.0 mg SEP-608 was conducted in methanol with 1 mol/L HCl at 50 ^{o}C for 2 hours. After extracted with chloroform, the aqueous layer was subjected to preparative HPLC (ZORBAX SB-Aq PrepHT, 7 μm, 21.2 mm × 250 mm, Agilent, Santa Clara, CA, USA) eluted with methanol/water (4/96, v/v) containing 0.1% trifluoroacetic acid at a flow rate of 10 mL/min. After lyophilizing, 5.6 mg SEP-327 was obtained.

Isolation of SEP-384. After centrifugation, the supernatant of 5 L *S. albus ⊿sepD* culture broth was concentrated and subjected to a Sephadex LH-20 column (35 mm × 700 mm). The water eluted fractions with SEP-384 were collected and lyophilized to yield a grey pale powder. After dissolved in a small volume of water, SEP-384 was refined by preparative HPLC (Zorbax SB-Aq PrepHT, 7 μm, 21.2 mm × 250 mm, Agilent, Santa Clara, CA, USA) eluted with water containing 0.1% trifluoroacetic acid at a flow rate of 5 mL/min. After lyophilizing, 2.3 mg SEP-384 was obtained.

Expression and purification of GmhA, GmhB, HldE, and HldD. The 0.6-kb fragment containing gene *gmhA* was amplified with primers GmhAU/GmhAD using *E. coli* TOP 10 genomic DNA as a template. After verified by sequencing, the fragment was digested with *Nde*I/*Bam*HI and inserted into the same sites of pET28a to generate

pET28a-GmhA. A single transformant of *E. coli* BL21 (DE3)/ pET28a-GmhA was inoculated into LB with 50 μg/mL kanamycin and cultured overnight at 37 ^{o}C, 220 rpm. The overnight culture was used to inoculate LB medium with 50 μg/mL kanamycin at 1:100 dilution and incubated at 37 ^{o}C, 220 rpm until OD_{600} reached 0.6. Expression of GmhA was then induced by the addition of isopropyl-β-thiogalactoside (IPTG) at a final concentration of 0.05 mM and cultured at 16 ^{o}C, 180 rpm for a further 16-18 hours.

GmhA was purified with Ni-NTA affinity column at 4°C following the manufacturer's instructions. After harvested by centrifugation, the cells were re-suspended in binding buffer (20 mM Tris-HCl, 500 mM NaCl, 5 mM imidazole, pH 7.9). After sonication, cell debris was removed by centrifugation at 16,000 × g for 30 min. The supernatant containing His_6-tagged proteins was loaded onto the Ni-NTA affinity column that had been equilibrated with binding buffer, then washed with wash buffer (binding buffer with 60 mM imidazole) followed by elution buffer (binding buffer with 250 mM imidazole). The fractions containing target protein were pooled and desalted by ultracentrifugation with an Amicon Ultra-4 10K centrifugal filter device. The purified protein was then concentrated by ultracentrifugation and stored at -80°C in 20 mM HEPES (pH 8.0) with 20% glycerol. Protein concentration was measured by the Bradford assay using bovine serum albumin as the standard.

The 0.6-kb fragment containing gene *gmhB* and the 0.9-kb fragment containing gene *hldD* were amplified with primer pairs GmhBU/GmhBD and HldDU/HldDD using *E. coli* TOP 10 genomic DNA as a template, respectively. , After verified by sequencing, the fragments were digested with *Nde*I/*Bam*HI and inserted into the same sites of pET28a respectively to generate pET28a-GmhB and pET28a-HldD. Similarly, the 1.4-kb fragment containing gene *hldE* was cloned with primer pair HldEU/HldED, verified by sequencing, digested with *Nhe*I/*Bam*HI, and inserted into the same sites of pET28a to afford pET28a-HldE. Protein expression and purification of GmhB, HldE, and HldD were performed using the same procedures as those for GmhA.

Expression and purification of SepB, SepL, and SepC. The 2.1-kb fragment containing gene *sepB* was amplified with primer pair SepBCU/SepBCD using *S. fimbriatus* CGMCC 4.1598 genomic DNA as a template. After verified by sequencing, the fragment was digested with *Nde*I/*Eco*RI and inserted into the same sites of pET28a to generate pET28a-SepB. Similarly, the 0.8-kb fragment containing gene *sepL* and the 0.9-kb fragment containing gene *sepC* were amplified with primer pairs SepLCU/SepLCD and SepCCU/SepCCD, verified by sequencing, digested with *Nde*I/*Bam*HI and inserted into the same sites of pET28a respectively to generate pET28a-SepL and pET28a-SepC. Protein expression and purification of SepB, SepL, and SepC were performed using the same procedures as those for GmhA.

To determine the cofactor used by SepC, the protein was denatured with $CHCl_3$. After centrifugation, the upper

aqueous was analyzed by analytic HPLC (Zorbax SB-Aq, 5 μm, 4.6 mm × 250 mm, Agilent, Santa Clara, CA, USA) eluted with acetonitrile/water (1/99, v/v) containing 0.1% trifluoroacetic acid at a flow rate of 1 mL/min with the detection UV wavelength at 260 nm.

Expression and purification of HygP. The 0.6-kb fragment containing gene *hygP* was amplified with primers HygPU/HygPD using *S. hygroscopicus* DSM 40578 genomic DNA as a template. After verified by sequencing, the fragment was digested with *Nde*I/*Bam*HI and inserted into the same sites of pET28a to afford pET28a-HygP. Protein expression and purification of HygP were performed with the same procedures as those for GmhA.

Preparation of ADP-D-*glycero*-*β*-D-*manno*-heptose. The ADP-D-*glycero*-*β*-D-*manno*-heptose standard was prepared by both enzymatic assay and organic synthesis methods. The enzymatic reaction of GmhA, GmhB, and HldE was carried out as previously described to prepare ADP-D-*glycero*-*β*-D-*manno*-heptose standard with minor modifications. The enzymes reacted in a 50 μL volume mixture containing 20 mM HEPES buffer (pH 8.0, adjusted with NaOH), 100 mM NaCl, 10 mM $MgCl_2$, 2 mM ATP, 0.2 mM D-sedoheptulose-7-phosphate and 5 μM GmhA, 5 μM GmhB, 5 μM HldE at 30 °C for 3 hours. The reaction was quenched by vibrating vigorously with an equal volume of chloroform and the chloroform layer was removed after centrifugation. HPLC analysis of the aqueous layer was carried out with a Dionex CarboPac^{TM} PA1 BioLC^{TM} column (4 × 250 mm, Thermo Fisher Scientific, Sunnyvale, CA, USA) on a Shimadzu HPLC system (Shimadzu, Kyoto, Japan). The column was developed with solvent A (ddH_2O) and solvent B (1.0 M CH_3COONH_4) at a flow rate of 1.0 mL/min. The percentage of solvent B stayed at 30% over 0-17 min, changed from 30% to 85% over 17-25 min, stayed at 85% over 25-30 min, and then decreased to 30% over 30-33 min. The detection wavelength was 260 nm. The HPLC fractions containing ADP-D-*glycero*-*β*-D-*manno*-heptose (retention time 16.5 min) were collected, verified by HR-MS and used as a standard.

The organic synthesis of ADP-D-*glycero*-*β*-D-*manno*-heptose was performed as described (Fig. S7) (6-8). During the process, compound **1** was characterized by MS and NMR to ensure the successful synthesis of D-*glycero*-D-*manno*-heptose (Table S4, Fig. S8). As we showed in Table S5, the ^{1}H NMR data of the synthesized ADP-D-*glycero*-*β*-D-*manno*-heptose was almost identical with the reported data.

Preparation of ADP-L-*glycero*-*β*-D-*manno*-heptose. To prepare the ADP-L-*glycero*-*β*-D-*manno*-heptose standard, the enzymatic reaction of GmhA, GmhB, HldE, and HldD was carried out in a 50 μL volume mixture containing 20 mM HEPES buffer (pH 8.0, adjusted with NaOH), 100 mM NaCl, 10 mM $MgCl_2$, 2 mM ATP, 0.2 mM D-sedoheptulose-7-phosphate, 5 μM GmhA, 5 μM GmhB, 5 μM HldE and 5 μM HldD at 30°C for 3 hours. The reaction was quenched by vibrating vigorously with an equal volume of chloroform. After centrifugation, the upper

aqueous phase was preserved for HPLC analysis as that for ADP-D-*glycero*-*β*-D-*manno*-heptose preparation. The HPLC fractions containing ADP-L-*glycero*-*β*-D-*manno*-heptose (retention time 17.5 min) were collected, verified by HR-MS and used as a standard.

Enzymatic assays of SepB and SepL. The enzymatic assay of SepB and SepL were set as well as that of GmhA, GmhB and HldE with slightly modification. The mixture containing 20 mM HEPES buffer (pH 8.0, adjusted with NaOH), 100 mM NaCl, 10 mM $MgCl_2$, 2 mM KCl, 2 mM ATP, 0.2 mM D-sedoheptulose-7-phosphate, 5 μM SepB and 5 μM SepL reacted at 30°C for 3 hours. To determine the sugar acceptor, 2 mM GTP, CTP, UTP, or dTTP was added to the reaction mixture together with 2 mM ATP. The hybrid assays were carried out the same as that of the SepB + SepL assay except that SepL was replaced by GmhB in the SepB + GmhB assay and SepB was replaced by GmhA and HldE in the GmhA + HldE + SepL assay. All the reactions were quenched by vibrating vigorously with an equal volume of chloroform. After centrifugation, the upper aqueous phases were analyzed by HPLC as that for ADP-D-*glycero*-*β*-D-*manno*-heptose preparation.

Enzymatic assays of SepC. The epimerase activity of SepC was checked using ADP-D-*glycero*-*β*-D-*manno*-heptose as a substrate. The reaction was carried out in a 50 μL volume mixture containing 20 mM HEPES buffer (pH 8.0, adjusted with NaOH), 100 mM NaCl, 0.2 mM ADP-D-*glycero*-*β*-D-*manno*-heptose, and 5 μM SepC at 30°C for 2 hours. The reaction was quenched by vibrating vigorously with an equal volume of chloroform. After centrifugation, the upper aqueous phase was analyzed by HPLC as that for ADP-D-*glycero*-*β*-D-*manno*-heptose preparation.

Enzymatic assays of HygP. The catalytic activity of HygP was studied in a hybrid assay together with GmhB and HldE. The assay was performed the same as that of the GmhA + GmhB + HldE assay except that GmhA was replaced by HygP. The reaction was quenched by vibrating vigorously with an equal volume of chloroform. After centrifugation, the upper aqueous phase was analyzed by HPLC as that for ADP-D-*glycero*-*β*-D-*manno*-heptose preparation.

Preparation of ADP-D-*glycero*-*β*-D-*altro*-heptose. ADP-D-*glycero*-*β*-D-*altro*-heptose was enzymatically synthesized with a scaled HygP, HldE and GmhB assay. Totally, a 200 mL solution containing 20 mM HEPES buffer (pH 8.0, adjusted with NaOH), 100 mM NaCl, 10 mM $MgCl_2$, 2 mM ATP, 1 mM D-sedoheptulose-7-phosphate, 43 μM HygP, 22 μM GmhB and 20 μM HldE was incubated at 30°C for 7 hours. The reaction mixture was filtrated with Millipore (Amicon Ultra-4, 10K MWCO) to remove the proteins. The product ADP-D-*glycero*-*β*-D-*altro*-heptose was isolated with a Dionex CarboPac™ PA1 BioLC™ Semi-Prep column (9 × 250 mm, Thermo Fisher Scientific, Sunnyvale, CA, USA) on a Shimadzu HPLC system (Shimadzu, Kyoto, Japan).

The column was developed with solvent A (ddH_2O) and solvent B (1.0 mol/L CH_3COONH_4) at a flow rate of 2.0 mL/min. The percentage of solvent B changed from 40-58% over 0-5min, stayed at 58% over 5-20min, changed from 58-90% over 20-22 min, and stayed at 90% over 22-30min. The detection wavelength was 260 nm. The product fraction (retention time at 20.1 min) was collected and evaporated in *vaccum* repeatedly to remove CH_3COONH_4. At last, ADP-D-*glycero*-*β*-D-*altro*-heptose was refined on a Sephadex LH20 column eluted with 50% methanol.

Spectroscopic analysis. LC-MS analyses were performed on an Agilent 1260/6460 Triple-Quadrupole LC/MS system (Santa Clara, CA, USA) with an electrospray ionization source. HR-ESI-MS was performed on an Agilent 1260 HPLC/6520 QTOF-MS instrument (Santa Clara, CA, USA). NMR spectra were recorded at room temperature on a Bruker-500 NMR spectrometer (Billerica, MA, USA).

Compound SEP-608. Pale powder; HR-ESI-MS(+) *m/z* 608.3768 $[M+H]^+$ (calcd for $C_{29}H_{49}N_7O_7$, 608.3766，$[M+H]^+$), see **Fig. S1*B***; ^1H and ^{13}C NMR data, see **Table S2**; ^1H and ^{13}C NMR spectra, see **Fig. S2-1*B*** and **S2-2*C***; ^1H-^1H COSY, HMQC and HMBC spectra, see **Fig. S2-2*D***, **S2-3*E*** and **S2-3*F***. The ^1H and ^{13}C NMR data are identical with those reported for septacidin analogs (9).

Compound SEP-327. Pale powder; HR-ESI-MS(+) *m/z* 327.1421 $[M+H]^+$ (calcd for $C_{12}H_{18}N_6O_5$, 327.1411，$[M+H]^+$), see **Fig. S3-1*B***; ^1H and ^{13}C NMR data, see **Table S3**; ^1H and ^{13}C NMR spectra, see **Fig. S3-1*C*** and **S3-2*D***; ^1H-^1H COSY, HMQC and HMBC spectra, see **Fig. S3-2*E***, **S3-3*F*** and **S3-3*G***.

Compound SEP-384. Pale powder; HR-ESI-MS(+) *m/z* 384.1639 $[M+H]^+$ (calcd for $C_{14}H_{21}N_7O_6$, 384.1626，$[M+H]^+$), see **Fig. S11*B***; ^1H and ^{13}C NMR data, see **Table S6**; ^1H and ^{13}C NMR spectra, see **Fig. S11*C*** and **S11*D***. Hydrolysis analyses, see **Fig. S12**.

ADP-D-*glycero*-*β*-D-*altro*-heptose. pale powder; HR-ESI-MS(-) *m/z* 618.0854 $[M-H]^-$ (calcd for $C_{17}H_{27}N_5O_{16}P_2$, 618.0855，$[M-H]^-$), see **Fig. S14-1*B***; ^1H and ^{13}C NMR data, see **Table S8**; ^1H and ^{13}C NMR spectra, see **Fig. S14-1*C***, **Fig. S14-2*D*** and **S14-2*E***; ^1H-^1H COSY, HMQC and HMBC spectra, see **Fig. S14-3*F***, **S14-4*G*** and **S14-4*H***; NOE difference spectrum and ^{31}P NMR spectrum, see **Fig. S14-5*I*** and **S14-5*J***.

Table S1. Homologous proteins and proposed functions of genes in the *sep* cluster.

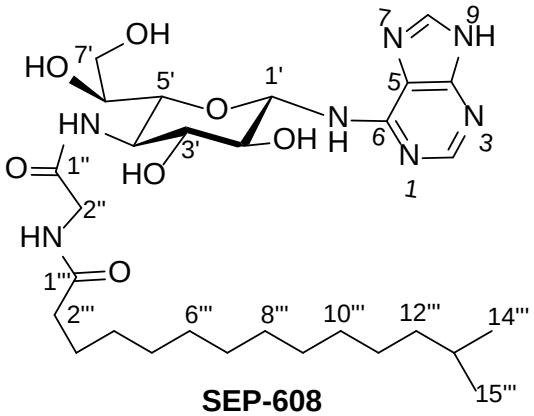
Genes	**Sizes(aa)**	**Proposed functions**	**Protein homologs* (identity/similarity)**
sepR	285	SARP family transcriptional regulator	Aur1PR3(ADM72849, 16/24)
sepA	174	hypothetical protein	(WP_079251747, 47/55)
sepB	707	D-sedoheptulose-7-phosphate isomerase (1-220 aa)	GmhA(NP_414757, 24/42)
		kinase and nucleotidyltransferase (221-707 aa)	HldE(NP_417524, 30/44)
sepC	320	epimerase	HldD(NP_418076, 27/42)
sepD	184	acyltransferase	(WP_025272485, 25/37)
sepE	341	glycosyltrasferase	RfaF(NP_418077, 18/29)
sepF	360	hypothetical protein	(KKR37865, 16/23)
sepG	415	aminotransferase	CetM(ACH85576, 57/67)
sepH	331	acyltransferase	(WP_075641650, 18/28)
sepI	510	oxidoreductase	StsB(CAH94317, 38/51)
sepJ	304	epimerase	(WP_077025002, 21/34)
sepK	423	MFS transporter	JadL(CCA59275, 23/36)
sepL	287	phosphatase	GmhB(NP_414742, 25/33)
sepM	366	hypothetical protein	(WP_086770645, 68/77)
sepN	243	hypothetical protein	(WP_059209674, 61/67)
sepO	545	GH3-superfamily protein	(AEC13081, 45/55)
sepP	775	HMG-CoA reductase	(SCD55657, 74/83)
sepQ	279	hypothetical protein	(WP_059209672, 54/65)

*Given in brackets are GenBank accession numbers and percentage of identity/percentage of similarity.

Table S2. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) data for compound SEP-608 in DMSO-d_6.*

No.	δ_H(ppm, *J*=Hz)	δ_C(ppm)	No.	δ_H(ppm, *J*=Hz)	δ_C(ppm)
1			1″		168.3
2	8.38(s)	151.1	2″	3.73(d, 6.0, 2H)	43.2
3			1‴		172.7
4		130.1	2‴	2.14(t, 7.5, 2H)	29.0
5		151.1	3‴	1.50(m, 2H)	27.2
6		156.3	4‴	1.28(m, 2H)	34.2
7			5‴	1.27(m, 2H)	34.3
8	8.32(s)	151.2	6‴	1.21(m, 2H)	32.3
1′	5.35(brs)	130.2	7‴	1.13(m, 2H)	38.7
2′	3.41(m)	72.9	8‴	1.11(m, 2H)	29.2
3′	3.55(m)	78.4	9‴	1.10(m, 2H)	29.3
4′	3.64(t, 10.0)	52.6	10‴	1.05(m, 2H)	36.5
5′	3.44(m)	75.4	11‴	1.03(m, 2H)	24.6
6′	3.52(m)	72.1	12‴	1.48(m, 2H)	25.6
7′	3.32(m, 2H)	62.4	13‴	1.49(m)	27.2
			14‴	0.82(d, 6.5, 3H)	19.3
			15‴	0.81(d, 6.5, 3H)	11.4

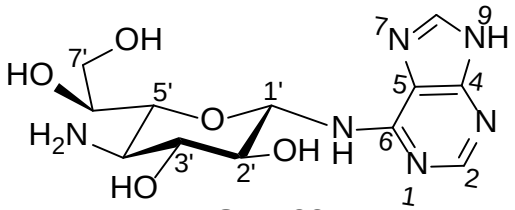
*****^{1}H NMR and ^{13}C NMR data of the aminoheptose moiety (from 1′ to **7**′) were assigned based on the HMBC and HMQC data.



SEP-608

Table S3. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) data for compound SEP-327 in D_2O.

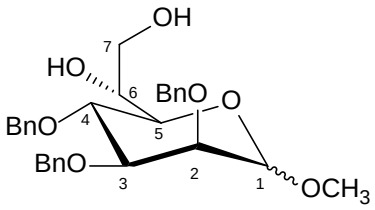
No.	δ_H(ppm, *J*=Hz)	δ_C(ppm)
2	8.20(s)	151.8
4		117.8
5		150.8
6		152.8
8	8.09(s)	141.2
1′	5.39(d, 9.0)	81.1
2′	3.67(t, 9.0)	72.0
3′	3.78(m)	73.1
4′	3.33(t,10.0)	54.8
5′	3.84(dd, 8.0 and 10.0)	71.2
6′	3.78(m)	73.1
7′	3.56(d, 2.5, 2H)	62.1



SEP-327

Table S4. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) data for compound **1** in CD_3OD.

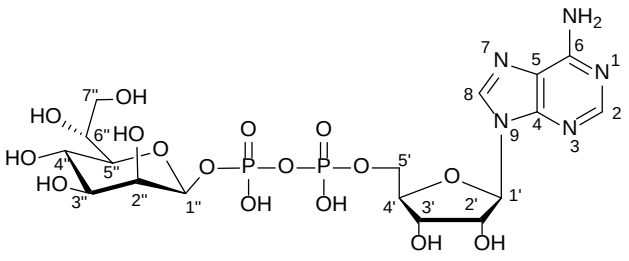
No.	δ_H (ppm, J=Hz)	δ_C (ppm)
1	4.75(d, 1.1)	98.8
2	3.85(d, 2.85)	80.1
3	3.83(m)	74.8
4	3.92(d, 9.55)	74.9
5	3.95(m)	72.3
6	3.68(dd, 3.28 and 9.85)	73.0
7	3.72,3.64(m, 2H)	62.5
1-OCH_3	3.35(s)	53.9
-OCH_2Ph	4.52-4.90(m, 6H)	71.4-75.0
Aromatic H	7.24-7.39(m, 15H)	127.2-128.0



Compound 1

Table S5. Comparison of ^{1}H NMR data for synthesized ADP-D-*glycero*-*β*-D-*manno*-heptose in D_2O with the reported data in literature (8).

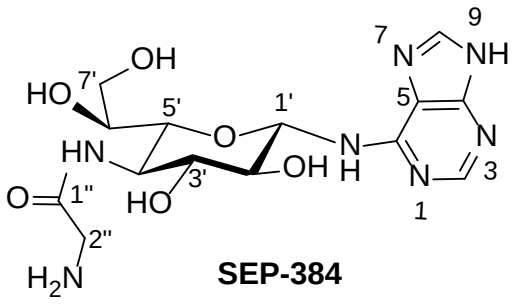
No.	Synthesized in this study δ_H（ppm, J=Hz）	Literature reported δ_H（ppm, *J*=Hz）
1		
2	8.51 (s)	8.53 (s)
3		
4		
5		
6		
7		
8	8.28 (s)	8.29 (s)
1′	6.16(d, 5.85)	6.16(d, 5.8)
2′	4.63(m)	4.73(m, 4.9)
3′	4.54(m)	4.54(dd, 3.8)
4′	4.41(m)	4.41(m)
5′	4.23(m, 2H)	4.23(m, 2H)
1″	5.21(d, 8.5)	5.21(dd, 1.0 and 8.7)
2″	4.06(d, 3.2)	4.07(dd, 3.3)
3″	3.64(dd, 3.2 and 9.3)	3.63(dd, 9.4)
4″	3.69(d, 9.3)	3.69(t, 9.4)
5″	3.45(dd, 3.1 and 9.75)	3.45(dd, 3.3)
6″	4.00(m)	3.99(m)
7″	3.75(m, 2H)	3.75(m, 2H)



ADP-D-*glycero*-β-D-*manno*-heptose

Table S6. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) data for compound SEP-384 in DMSO-d_6.

No.	δ_H (ppm, J=Hz)	δ_C(ppm)
2	8.45(s)	148.6
4		115.1
5		148.9
6		152.2
8	8.35(s)	143.2
1′	5.44 (brs)	81.3
2′	3.63(m)	74.5
3′	3.68(m)	71.9
4′	3.92(t, 10.0)	52.1
5′	3.75(dd, 8.0 and 10.0)	70.7
6′	3.81(m)	77.1
7′	3.69(d, 2.5, 2H)	61.9
1″		167.3
2″	3.80(m, 2H)	40.5



SEP-384

Table S7. Homologous proteins and proposed functions of genes in the *hyg* cluster.

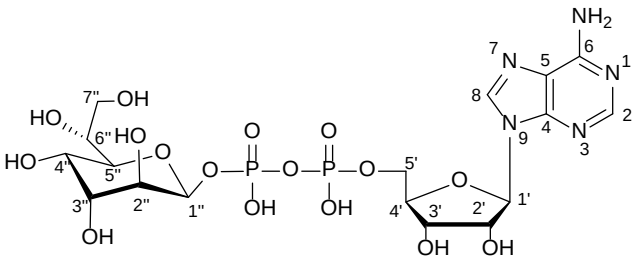
Genes	**Sizes(aa)**	**Proposed functions**	**Protein homologs* (identity/similarity)**
hygV	616	hygromycin B exporter	MdlA(P77265, 25/41)
hygW	604	hygromycin B exporter	MdlA(P77265, 26/42)
hygA	332	aminoglycoside *O*-phosphotransferase	APH(WP_067081005, 99/99)
hygK	319	NDP-heptose/hexose epimerase	GalE(P09147, 36/49)
hygU	162	phosphatase	GmhB(NP_414742, 29/41)
hygD	401	glycosyltransferase	AmgK(ACR82904, 68/76)
hygO	287	nucleotidyltransferase	GalT(NP_415279, 22/31)
hygE	339	2-deoxy-*scyllo*-inosamine dehydrogenase	NeoA(BAD95818, 42/54)
hygP	198	phosphoheptose isomerase	GmhA(NP_414757, 27/46)
hygX	258	oxidase/hydroxylase	AmgI(ACR82902, 67/81)
hygL	377	NDP-heptose aminotransferase	AmgH(ACR82901, 65/76)
hygJ	308	NDP-heptose/hexose epimerase	GalE(P09147, 23/36)
hygY	343	oxidoreductase	SpeY(ABW87805, 41/53)
hygF	270	aminocyclitol galactosyltransferase	SpcF(AAD45551, 45/54)
hygN	351	kinase	HddA(AAK27850, 25/44)
hygC	410	2-deoxy-*scyllo*-inosose synthase	BtrC(Q9S5E2, 34/52)
hygM	272	*N*-methyltransferase	AmgB(ACR82895,56/64)
hygS	434	2-deoxy-*scyllo*-inosose aminotransferase	BtrR(Q8G8Y2, 36/50)
hygT	182	hypothetical protein	(WP_078509916, 30/33)
hygI	163	transcriptional regulator	KanI(CAF31582,56/66)
hygH	168	hypothetical protein	(WP_067082097, 90/91)
hygG	392	hypothetical protein	(WP_067082100, 99/99)
hygZ	662	transposase	(WP_053711732, 85/88)

*Given in brackets are GenBank accession numbers and percentage of identity/percentage of similarity

Table S8. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) data for ADP-D-*glycero*-*β*-D-*altro*-heptose in D_2O.

No.	δ_H (ppm, *J*=Hz)	δ_C(ppm)
1		
2	8.10(s)	152.0
3		
4		149.0
5		118.8
6		155.6
7		
8	8.38(s)	138.3
1′	6.05(d, 5.5)	86.6
2′	4.67(m)[a]	74.4
3′	4.45(t, 4.5)	70.4
4′	4.31(brs)	83.7
5′	4.15(2H, m)	65.2
1″	4.88(t, 7.5)	97.9
2″	3.30(bt, 7.5)	73.3
3″	3.43(m)[a]	75.3
4″	3.42(m)[a]	70.1
5″	3.47(dd, 2.5 and 9.5)	76.7
6″	3.91(m)	72.4
7″	3.63(br, 2H)	61.5

Notes: [a] Observed from HMQC.



ADP-D-*glycero*-β-D-*altro*-heptose

Table S9. Bacterial strains and plasmids used in this study.

Strains or plasmids	Characteristics*	Sources
Escherichia coli		
Top10	General cloning host	Invitrogen
BW25113	Strain for λ RED-mediated PCR-targeting mutagenesis	(2)
ET12567	Strain deficient in DNA methylation	(4)
ET12567/pUZ8002	Donor strain for *E. coli-Streptomyces* conjugation	(4)
BL21 (DE3)	Host strain for protein expression	Novagen
Streptomyces		
S. fimbriatus CGMCC 4.1598	Wild-type septacidin producer	CGMCC
S. hygroscopicus DSM 40578	Hygromycin B producer	CGMCC
S. albus J1074	Heterologous expression host for septacidin	(4)
S. albus 153	*S. albus* J1074/pSET153, the negative control	this study
S. albus 1598	*S. albus* J1074/pSET1598, septacidin heterologous expression strain	this study
S. albus ΔsepR	*S. albus* J1074/pSET1598-*ΔsepR*, the Δ*sepR* mutant	this study
S. albus ΔsepB	*S. albus* J1074/pSET1598-*ΔsepB*, the Δ*sepB* mutant	this study
S. albus ΔsepC	*S. albus* J1074/pSET1598-*ΔsepC*, the Δ*sepC* mutant	this study
S. albus ΔsepD	*S. albus* J1074/pSET1598-*ΔsepD*, the Δ*sepD* mutant	this study
S. albus ΔsepH	*S. albus* J1074/pSET1598-*ΔsepH*, the Δ*sepH* mutant	this study
S. albus ΔsepL	*S. albus* J1074/pSET1598-*ΔsepL*, the Δ*sepL* mutant	this study
S. albus ΔsepMNOPQ	*S. albus* J1074/pSET1598-*ΔsepMNOPQ*, the Δ*sepMNOPQ* mutant	this study
Plasmids		
pET28a	Kan^r, protein expression vector	Novagen
pUK-T5promoter	Kan^r, containing kanamycin resistant gene *aph(3')*-II	(10)
pSET153	Apr^r, ΦC31 int, p15A origin vector	(5)
pSET1598	Apr^r, pSET153 derivative harboring the whole *sep* cluster	this study
pSET1598-*ΔsepR*	Apr^r, pSET1598 derivative with *sepR* in-frame deletion	this study
pSET1598-*ΔsepB*	Apr^r, pSET1598 derivative with *sepB* in-frame deletion	this study
pSET1598-*ΔsepC*	Apr^r, pSET1598 derivative with *sepC* in-frame deletion	this study
pSET1598-*ΔsepD*	Apr^r, pSET1598 derivative with *sepD* in-frame deletion	this study
pSET1598-*ΔsepH*	Apr^r, pSET1598 derivative with *sepH* in-frame deletion	this study
pSET1598-*ΔsepL*	Apr^r, pSET1598 derivative with *sepL* in-frame deletion	this study
pSET1598-*ΔsepMNOPQ*	Apr^r, pSET1598 derivative with *sepMNOPQ* in-frame deletion	this study
pET28a-GmhA	Kan^r, pET28a derivative for the expression of *gmhA* gene	this study
pET28a-GmhB	Kan^r, pET28a derivative for the expression of *gmhB* gene	this study
pET28a-HldE	Kan^r, pET28a derivative for the expression of *hldE* gene	this study
pET28a-HldD	Kan^r, pET28a derivative for the expression of *hldD* gene	this study
pET28a-SepB	Kan^r, pET28a derivative for the expression of *sepB* gene	this study
pET28a-SepC	Kan^r, pET28a derivative for the expression of *sepC* gene	this study
pET28a-SepL	Kan^r, pET28a derivative for the expression of *sepL* gene	this study
pET28a-HygP	Kan^r, pET28a derivative for the expression of *hygP* gene	this study

*Apr^r, apramycin resistance; Kan^r, kanamycin resistance.

Table S10. Primers used in this study.

Primer names	Sequences (5'-3')*	Restriction sites
SepRU	acggaaggaaaacagattatggccgaaccttacggcgccACTAGTgaacttcaagatcccctcac	*Spe*I
SepRD	ccccgccccatgatgagcttgacgaccgcctcaccacatCCTAGGtcagagcgcttttgaagctg	*Bln*I
SepBU	gacagcgacagtaggagcaccgacatgacgtccgacaccACTAGTgaacttcaagatcccctcac	*Spe*I
SepBD	tcacagcatccgtgccgattcgatcagcgacgacgtcgaCCTAGGtcagagcgcttttgaagctg	*Bln*I
SepCU	cggaccgcagaggtggcaggcatgagccggcagagcgtgACTAGTgaacttcaagatcccctcac	*Spe*I
SepCD	cagctcatgggcagcgctccgcagatcgtccgggcccggCCTAGGtcagagcgcttttgaagctg	*Bln*I
SepDU	ggatatcacgtgagtcttgacatggtcgtggtgggcaagACTAGTgaacttcaagatcccctcac	*Spe*I
SepDD	cgcgccgtgaaagacgcgtcagtcatcggctcccggtcgCCTAGGtcagagcgcttttgaagctg	*Bln*I
SepHU	gtcgcggcgaaagaggcgcaccggatgaacgccatcgcgACTAGTgaacttcaagatcccctcac	*Spe*I
SepHD	tcacaccgggctcggctgctcccggctcgccgtcacggtCCTAGGtcagagcgcttttgaagctg	*Bln*I
SepLU	ggagaacgcgccctctccgagcccgccgcggtcatgagcACTAGTgaacttcaagatcccctcac	*Spe*I
SepLD	gacgcgtcaggattcacgggacggccccttctgcacgtggCCTAGGtcagagcgcttttgaagctg	*Bln*I
SepMQU	ccgcccggcccgggggccatgacagaactcgtggtggcaACTAGTgaacttcaagatcccctcac	*Spe*I
SepMQD	ccgtcaccaggtgtccaggcgggctcctcgccgaccctgCCTAGGtcagagcgcttttgaagctg	*Bln*I
GmhAU	ggaattcCATATGtaccaggatcttattcgtaacg	*Nde*I
GmhAD	cgcGGATCCttacttaaccatctctttttcaatcaac	*Bam*HI
GmhBU	ggaattcCATATGgcgaagagcgtaccc	*Nde*I
GmhBD	cgcGGATCCtcattgtgccggtttttgctgc	*Bam*HI
HldEU	ctaGCTAGCatgaaagtaacgctgccagagtttg	*Nhe*I
HldED	cgcGGATCCttagccttttttatcctgttggatcttcttgatg	*Bam*HI
HldDU	ggaattcCATATGatcatcgttaccggcg	*Nde*I
HldDD	cgGGATCCttatgcgtcgcgattcag	*Bam*HI
SepBCU	ggaattcCATATGacgtccgacaccttgat	*Nde*I
SepBCD	cgGAATTCggaggggggtgtcgtcatc	*Eco*RI
SepLCU	ggaattcCATATGagcggcggcccgctcga	*Nde*I
SepLCD	cgGGATCCgacgcgtcaggattcacggga	*Bam*HI
SepCCU	ggaattcCATATGagccggcagagcgtg	*Nde*I
SepCCD	cgGGATCCcggacgcgctttcagatg	*Bam*HI
HygPU	ggaattcCATATGacacaggatttcttagccc	*Nde*I
HygPD	cgGGATCCtccggccggttcaccgct	*Bam*HI
guide RNA-F	gttttagagctagaaatagcaagttaaaataaggctagtc	
guide RNA-R	aaaagcaccgactcggtgccacttttcaagttgataacggactagccttattttaact	
sep-gF-P	taatacgactcactataggtccgcgtccgtcatgtccgagttttagagctagaaatagcaa	
sep-gR-P	taatacgactcactataggcgatcgagagcgtgaacgcagttttagagctagaaatagcaa	
sep-p15A-F	gcgagcgcggacggccttgcggatccgtgctagatccttttggttcatgtgcagctc	
sep-p15A-R	cacccggagcgtgttcggcggtatccctcgggtgaagatccttttgataatctcatg	

*The designed restriction site in each primer is capitalized and underlined.

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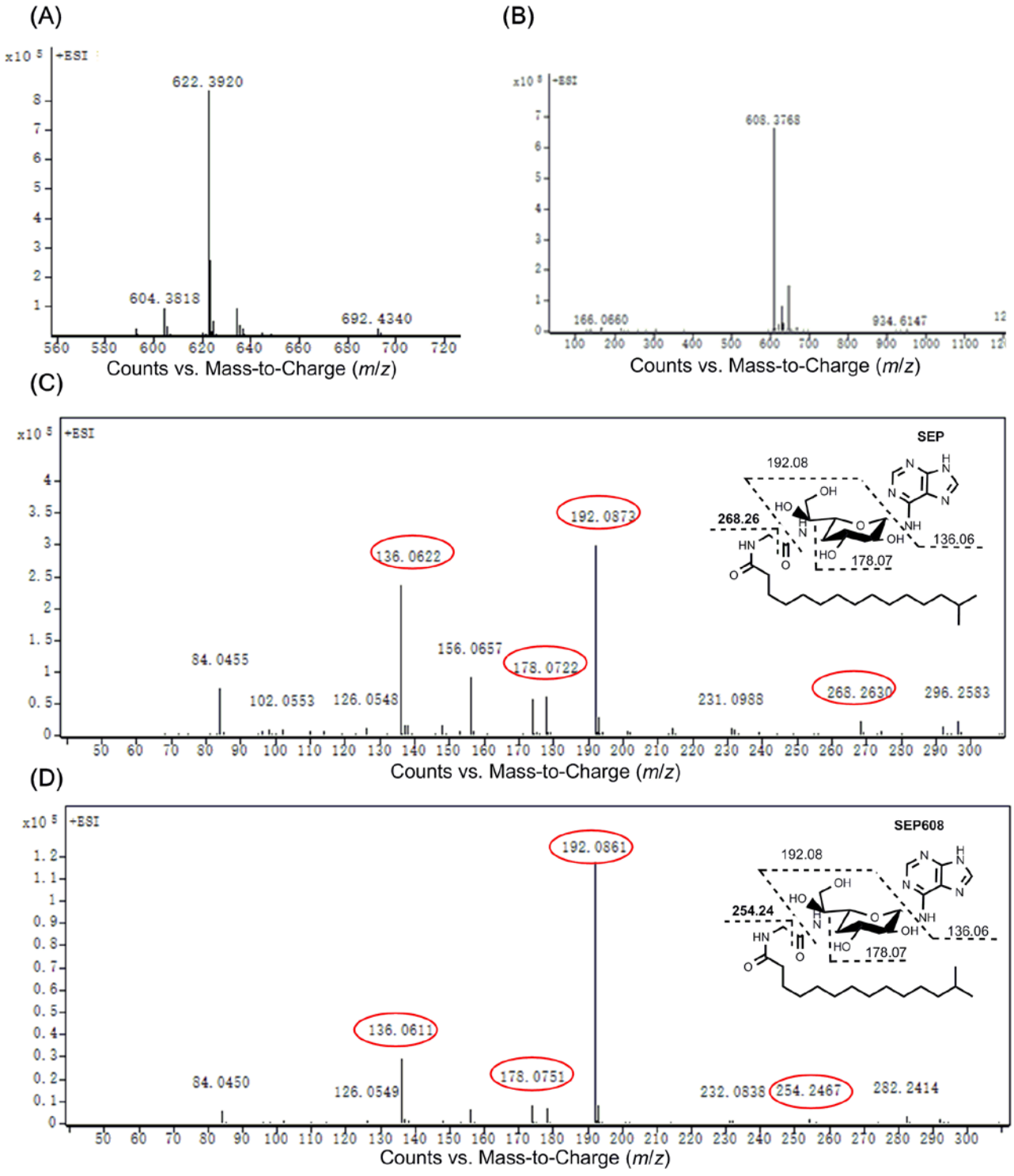

Figure S1. HR-MS and tandem MS analyses of septacidin and SEP-608 produced by the *sep* gene cluster heterologous expressed strain *S. albus* 1598**. (A)** HR-ESI-MS of septacidin, *m/z* 622.3920 $[M+H]^+$ (calcd for $C_{30}H_{51}N_7O_7$, 622.3923，$[M+H]^+$); **(B)** HR-ESI-MS of SEP-608, *m/z* 608.3768 $[M+H]^+$ (calcd for $C_{29}H_{49}N_7O_7$, 608.3766，$[M+H]^+$); **(C)** MS/MS spectrum and fragmentation pattern of septacidin; **(D)** MS/MS spectrum and fragmentation pattern of SEP-608.

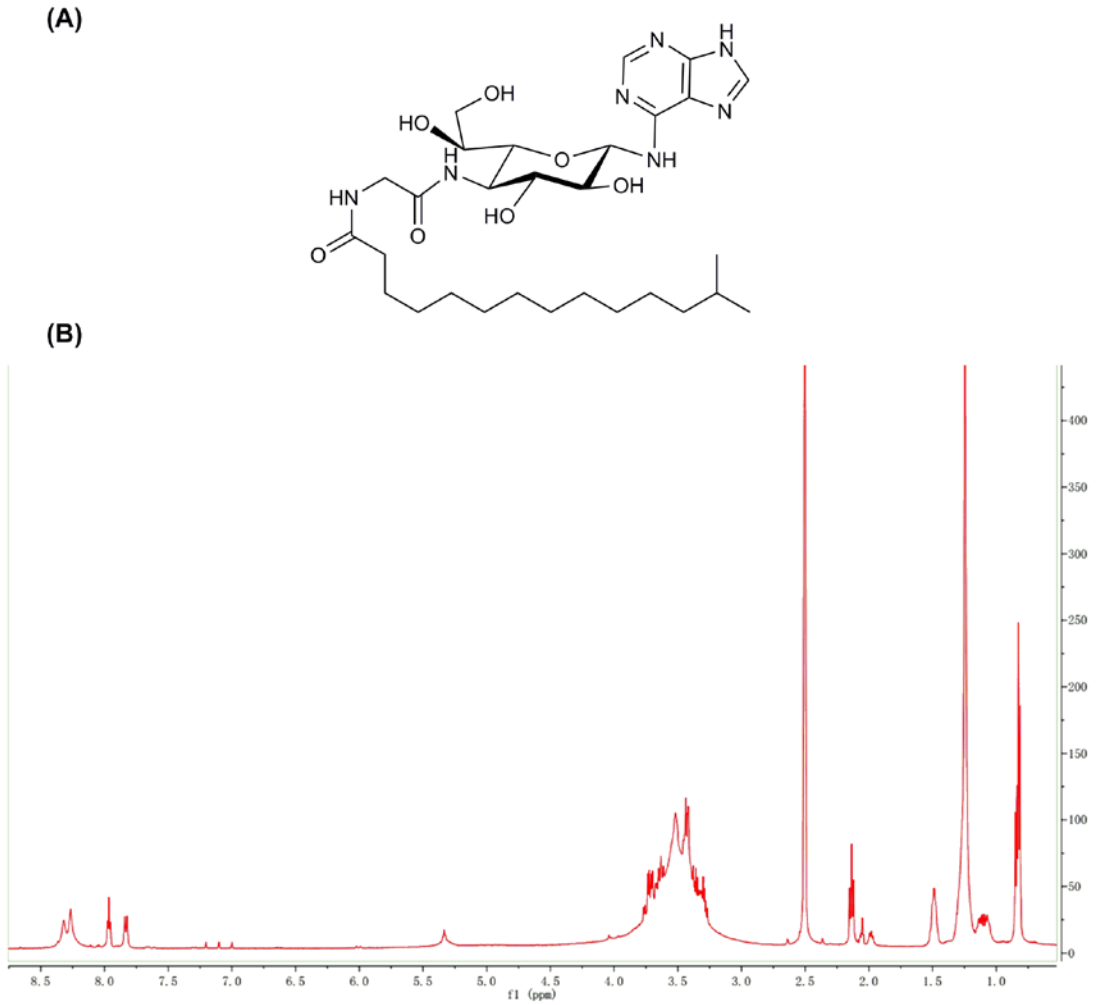

Figure S2-1. Spectral data of compound SEP-608. (**A**) Structure of compound SEP-608. (**B**) ^{1}H NMR spectrum of compound SEP-608 in DMSO-d_6.

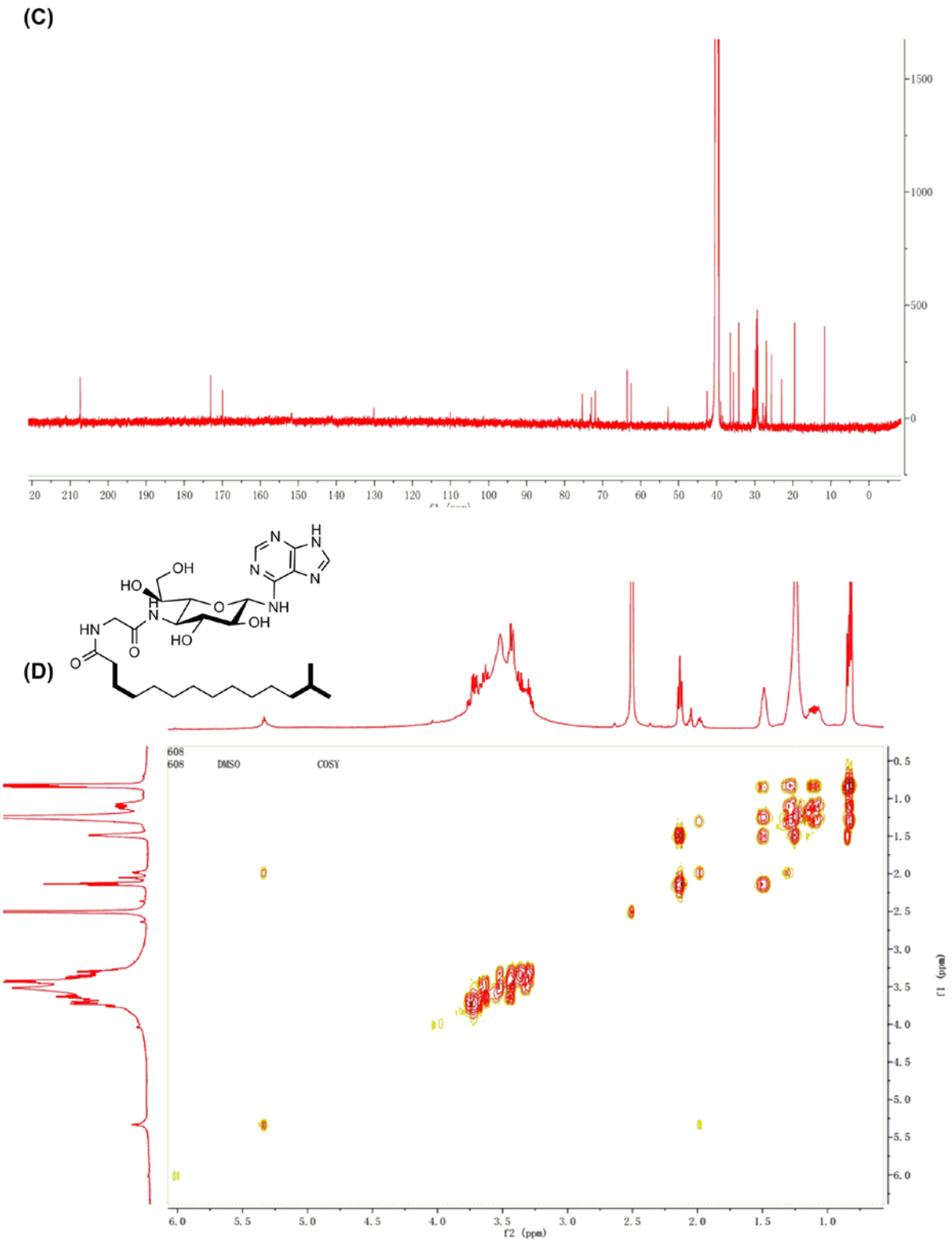

Figure S2-2. Spectral data of compound SEP-608. (**C**) ^{13}C NMR spectrum of compound SEP-608. (**D**) ^{1}H-^{1}H COSY spectrum of compound SEP-608.

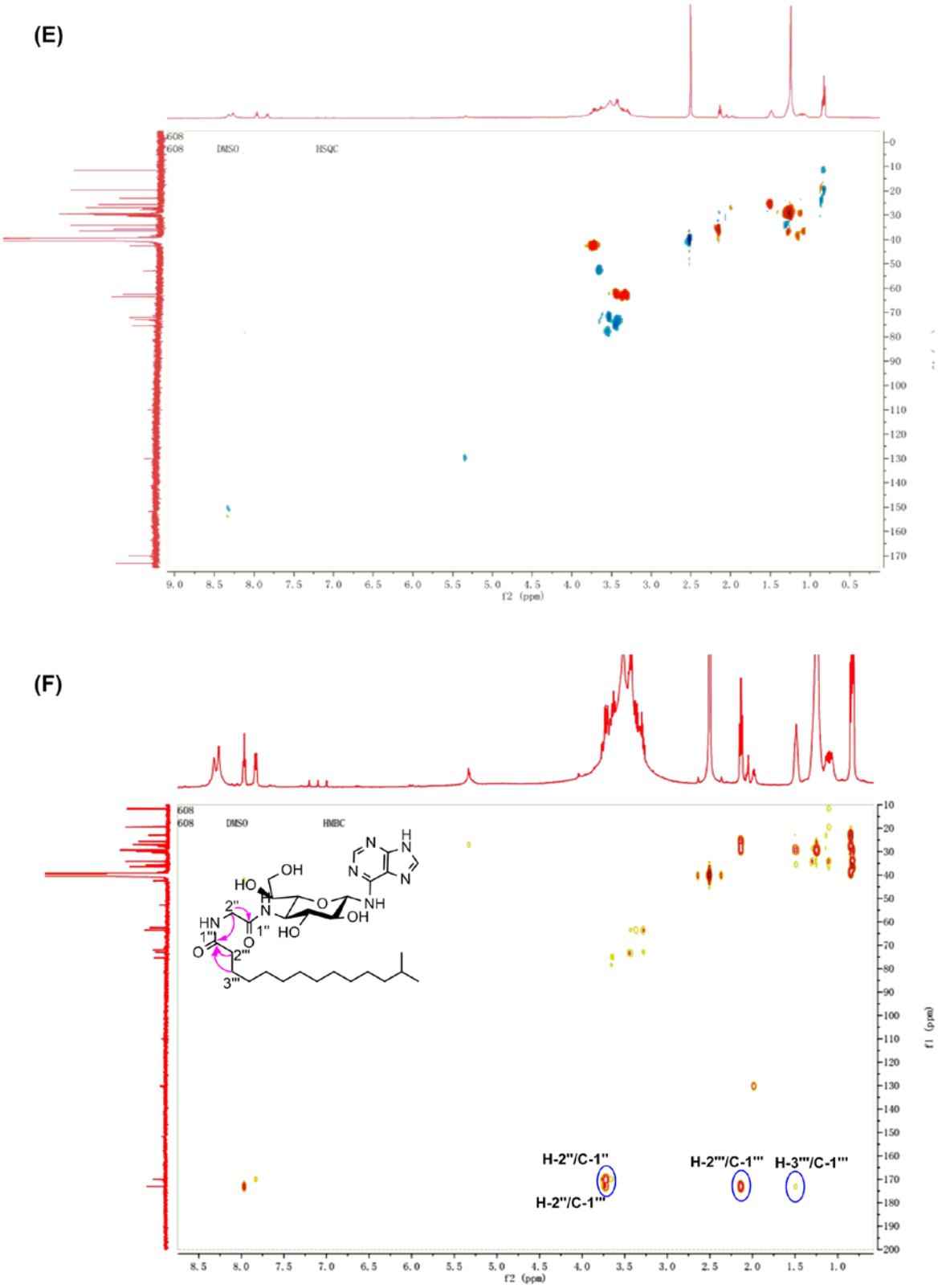

Figure S2-3. Spectral data of compound SEP-608. **(E)** HMQC spectrum of compound SEP-608. **(F)** HMBC spectrum of compound SEP-608.

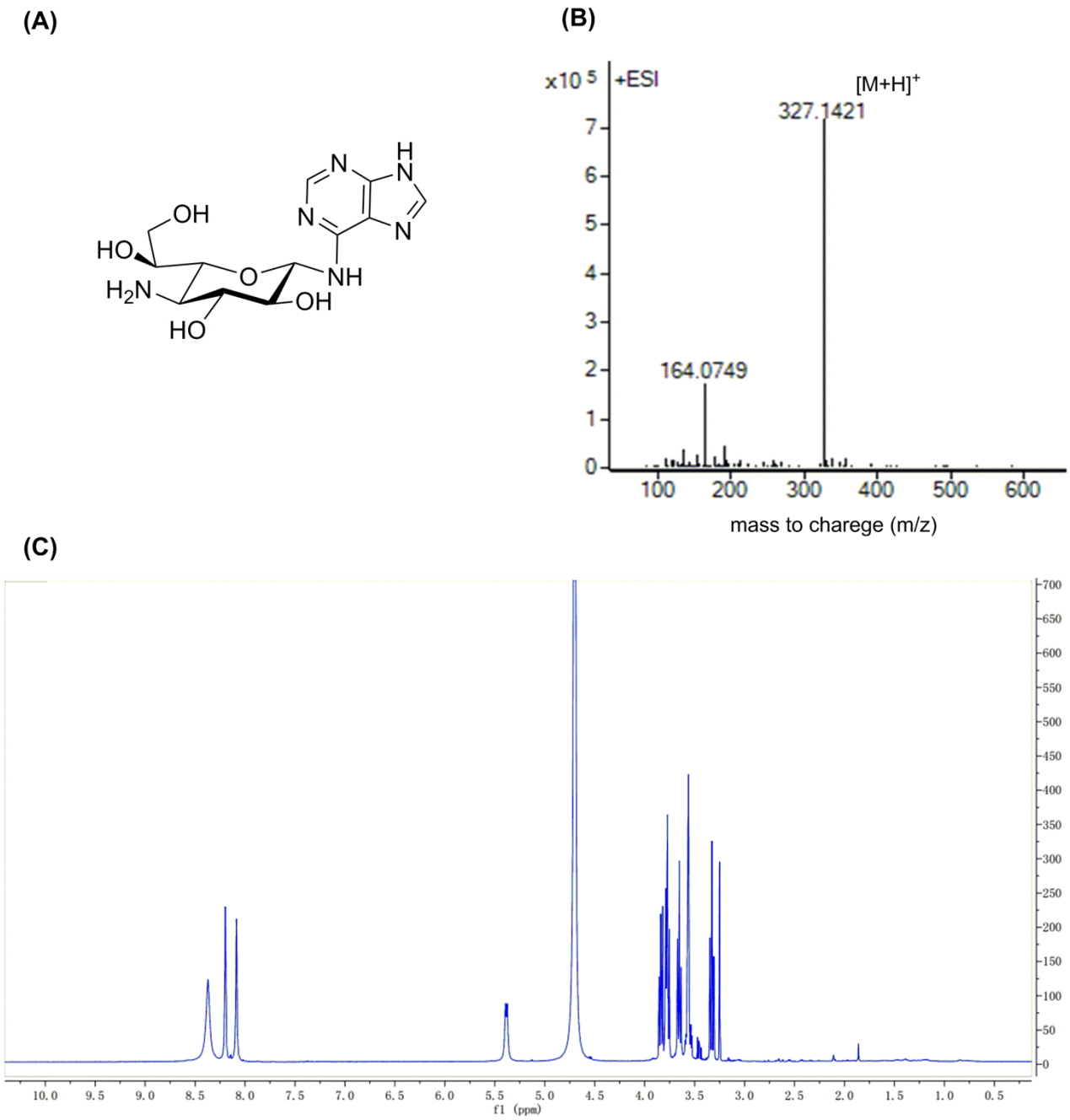

Figure S3-1. Spectral data of compound SEP-327. (**A**) Structure of compound SEP-327. (**B**) HR-ESI-MS spectrum of compound SEP-327. (**C**) ^{1}H NMR spectrum of compound SEP-327 in D_2O.

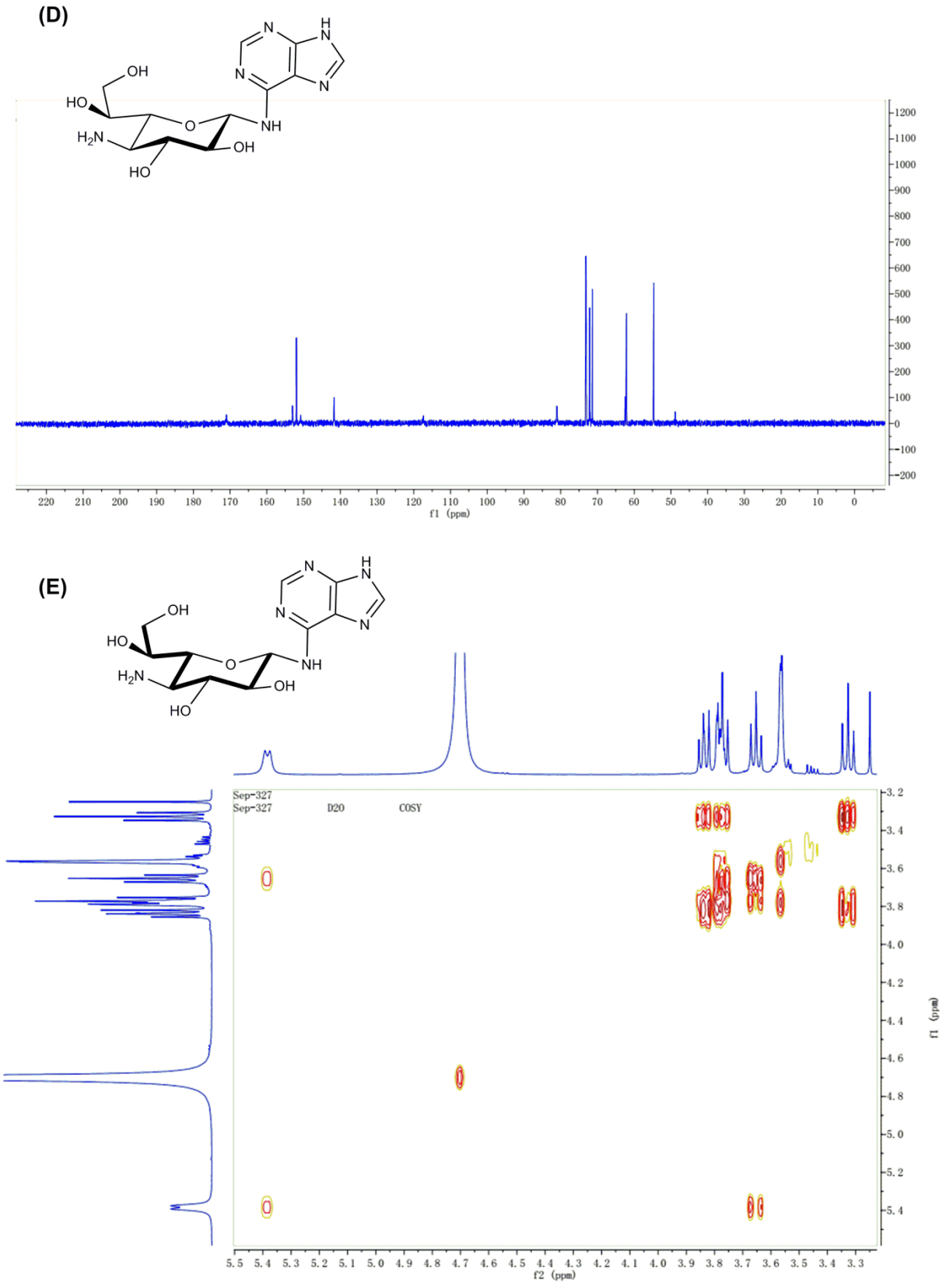

Figure S3-2. Spectral data of compound SEP-327. **(D)** ^{13}C NMR spectrum of compound SEP-327. **(E)** ^{1}H-^{1}H COSY spectrum of compound SEP-327.

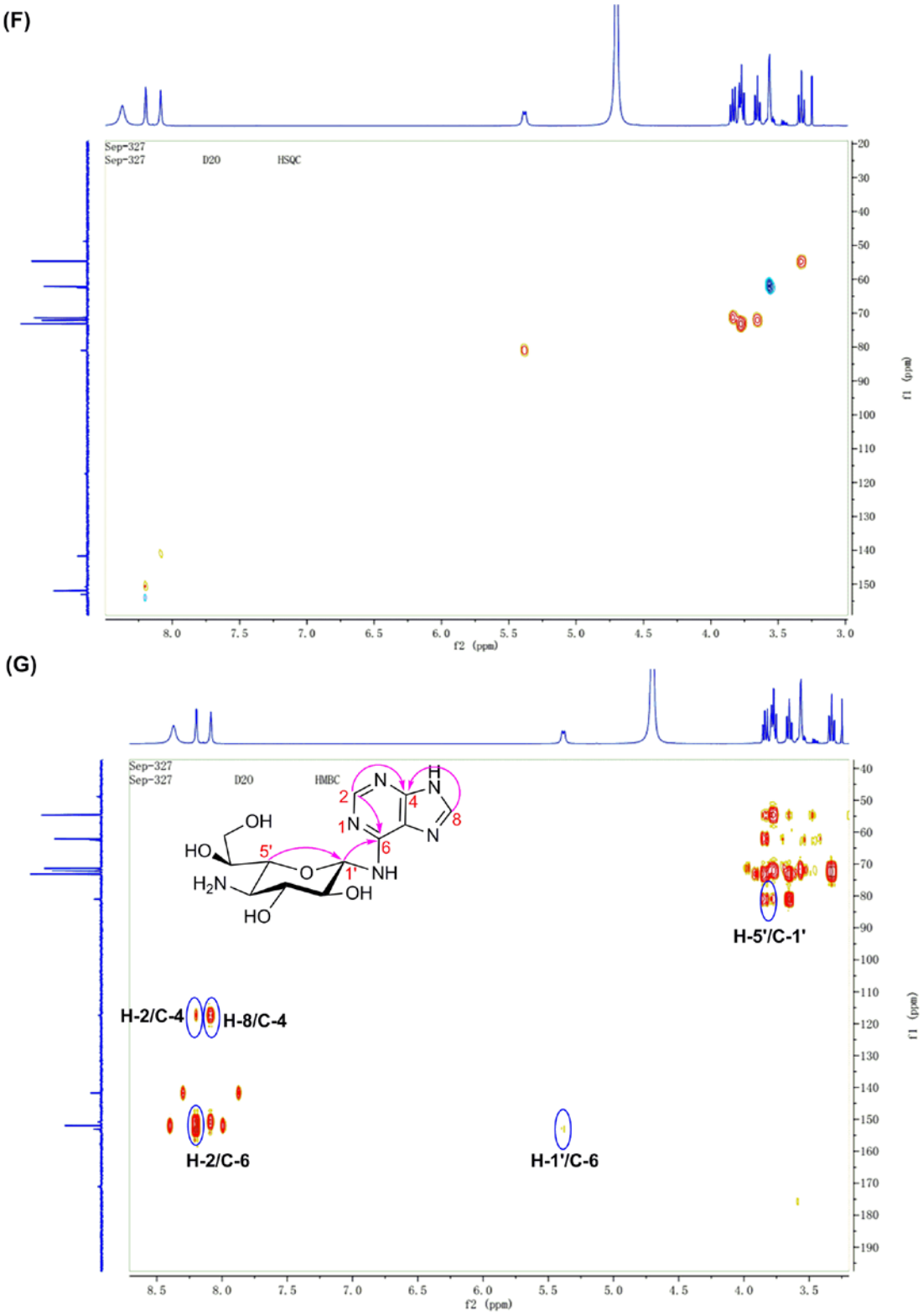

Figure S3-3. Spectral data of compound SEP-327. **(F)** HMQC spectrum of compound SEP-327. **(G)** HMBC spectrum of compound SEP-327.

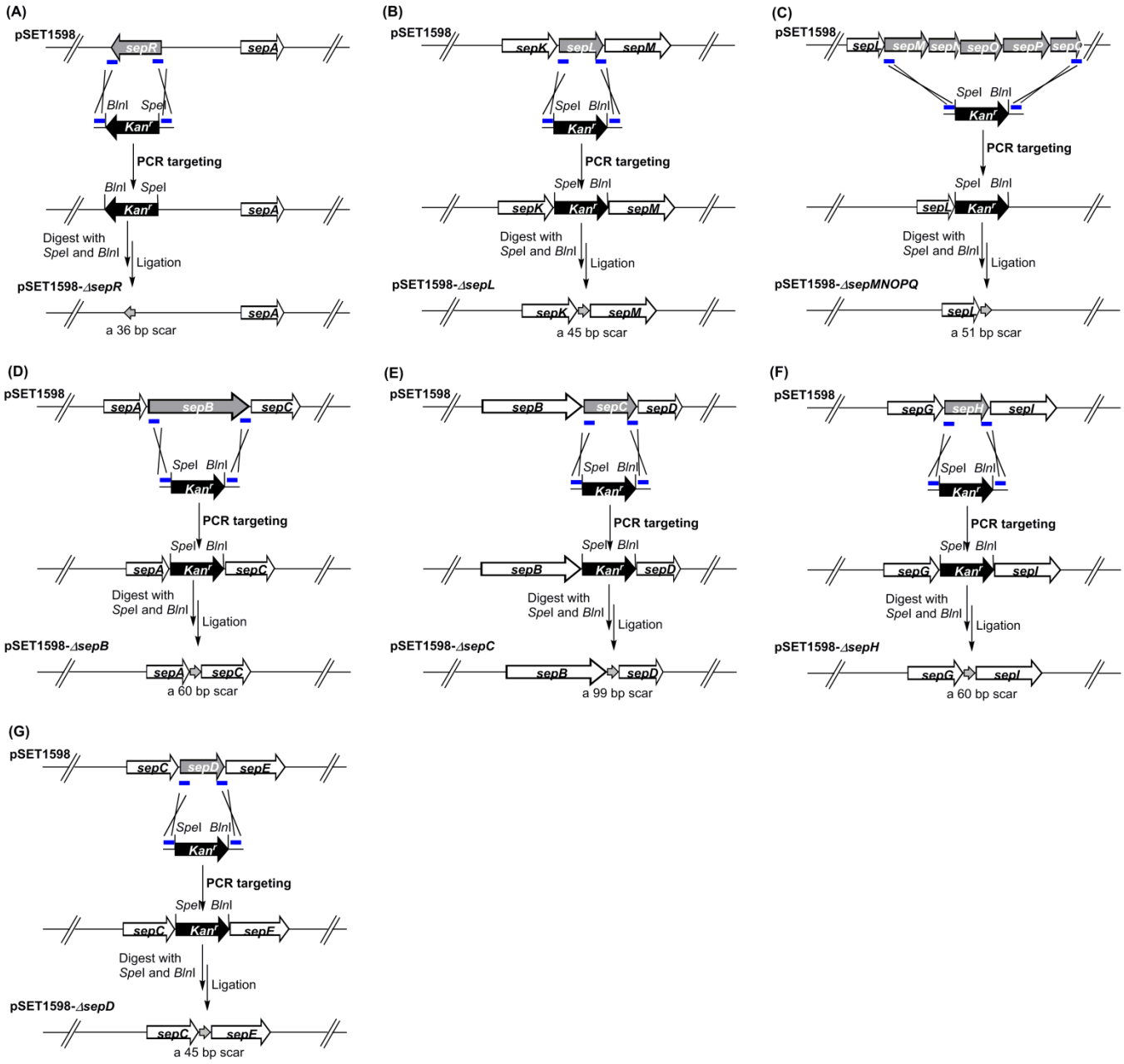

Figure S4. Construction of the *sep* gene cluster heterologous expression plasmids with different in-frame gene deletions. The *sep* gene in-frame deletion mutant strains were generated by introduction of these plasmids into the heterologous expression host *S. albus* J1074. **(A)** pSET1598-Δ*sepR*, *sepR* in-frame deleted; **(B)** pSET1598-Δ*sepL*, *sepL* in-frame deleted; **(C)** pSET1598-Δ*sepMNOPQ*, *sepMNOPQ* in-frame deleted together; **(D)** pSET1598-Δ*sepB*, *sepB* in-frame deleted; **(E)** pSET1598-Δ*sepC*, *sepC* in-frame deleted; **(F)** pSET1598-Δ*sepH*, *sepH* in-frame deleted; **(G)** pSET1598-Δ*sepD*, *sepD* in-frame deleted. The homologous sequences used for λ RED-mediated recombination are indicated with blue squares.

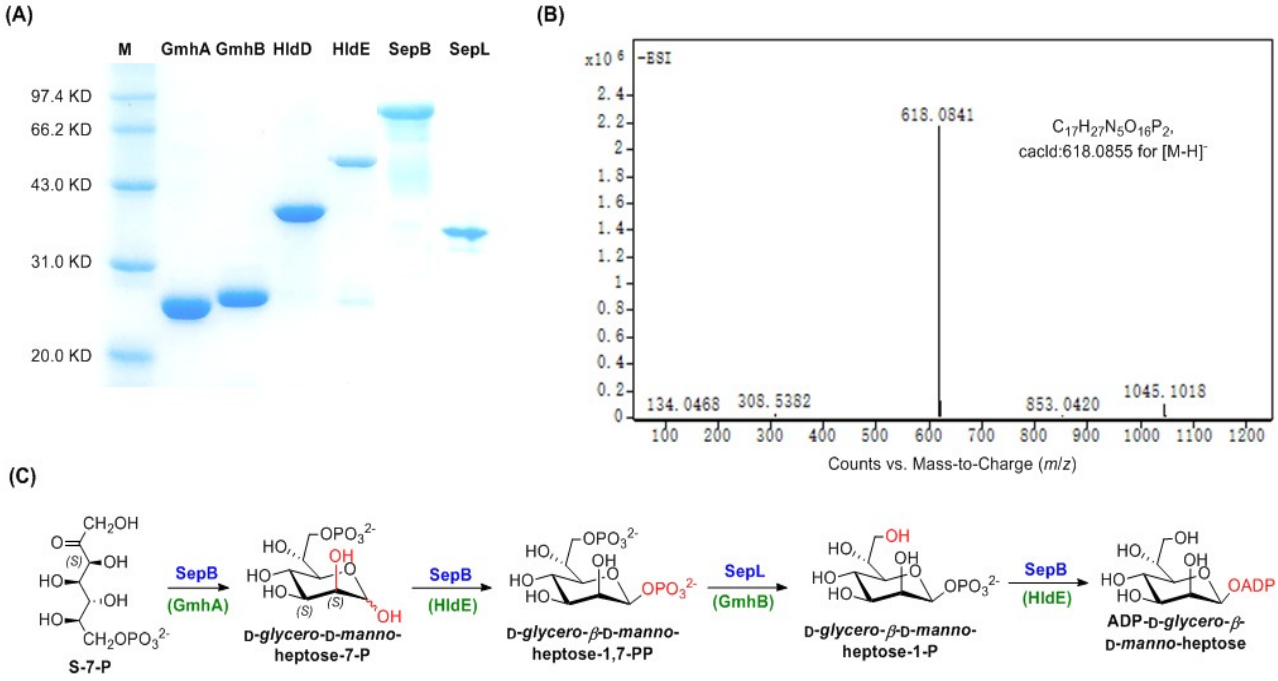

Figure S5. *In vitro* characterization of SepB and SepL. **(A)** SDS-PAGE analysis of GmhA, GmhB, HldD, HldE, SepB and SepL. Lane M, protein marker**. (B)** HR-ESI-MS spectrum of ADP-D-*glycero*-*β*-D-*manno*-heptose generated by the SepB + SepL assay. **(C)** ADP-D-*glycero*-*β*-D-*manno*-heptose biosynthetic pathway catalyzed by SepB and SepL. The isozymes involved in Gram-negative bacteria LPS heptose biosynthesis are bracketed and indicated with green.

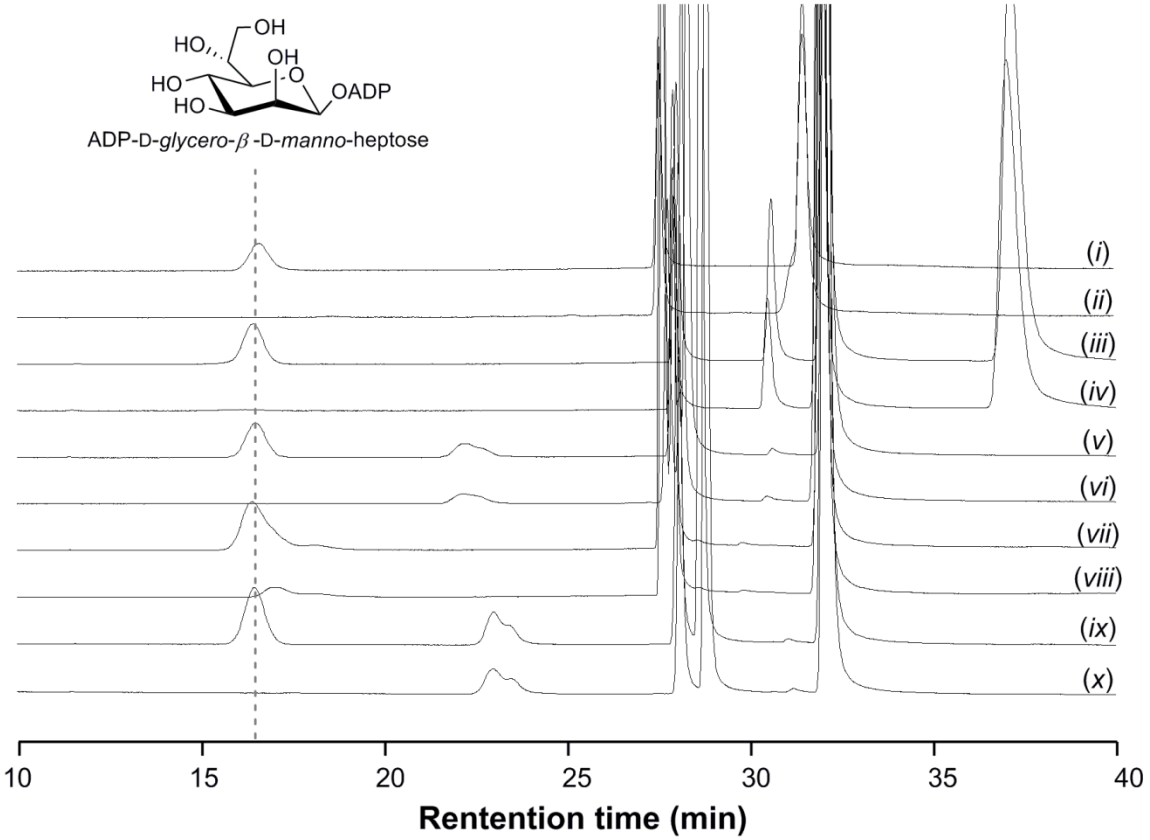

Figure S6. Enzymatic assays of SepB + SepL with different NTPs. (*i*) ATP; (*ii*) ATP negative control without substrate S-7-P; (*iii*) ATP + GTP; (*iv*)ATP + GTP negative control without substrate S-7-P; (*v*) ATP + dTTP; (*vi*) ATP + dTTP negative control without substrate S-7-P; (*vii*) ATP + CTP; (*viii*) ATP + CTP negative control without substrate S-7-P; (*ix*) ATP + UTP; (*x*) ATP+UTP negative control without substrate S-7-P.

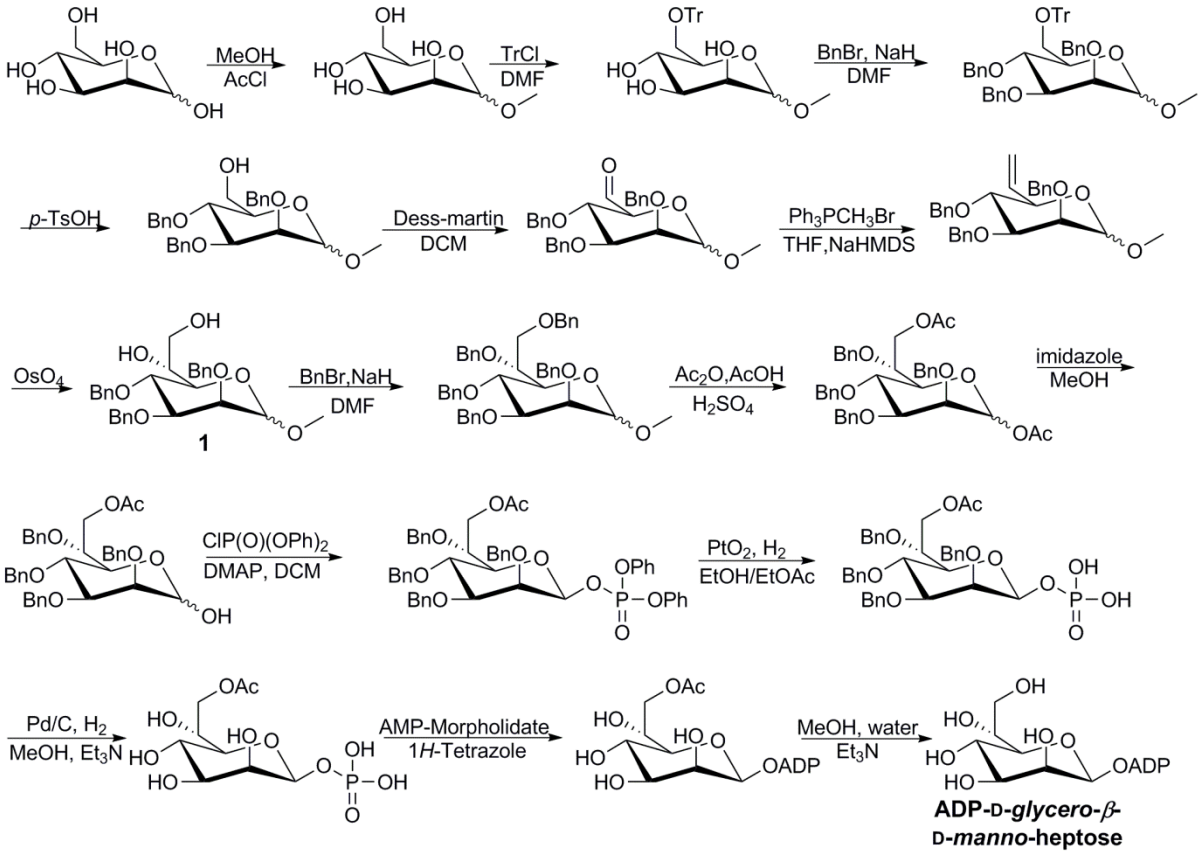

Figure S7. Synthesis route of ADP-D-*glycero*-*β*-D-*manno*-heptose.

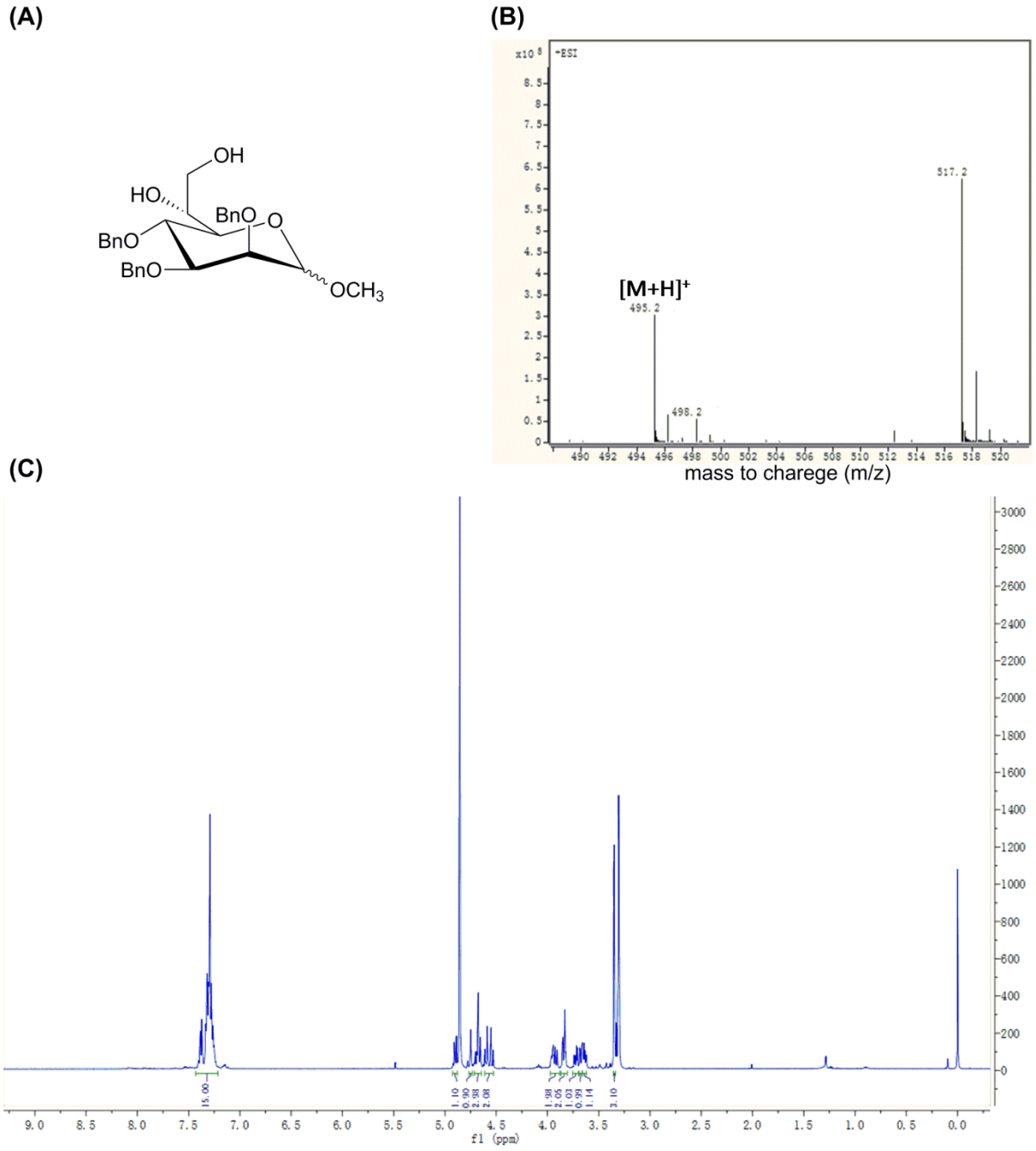

Figure S8-1. Spectral data of compound **1**. (A) Structure of compound **1**. (B) ESI-MS spectrum of compound **1**. (**C**) ^{1}H NMR spectrum of compound **1** in CD_3OD.

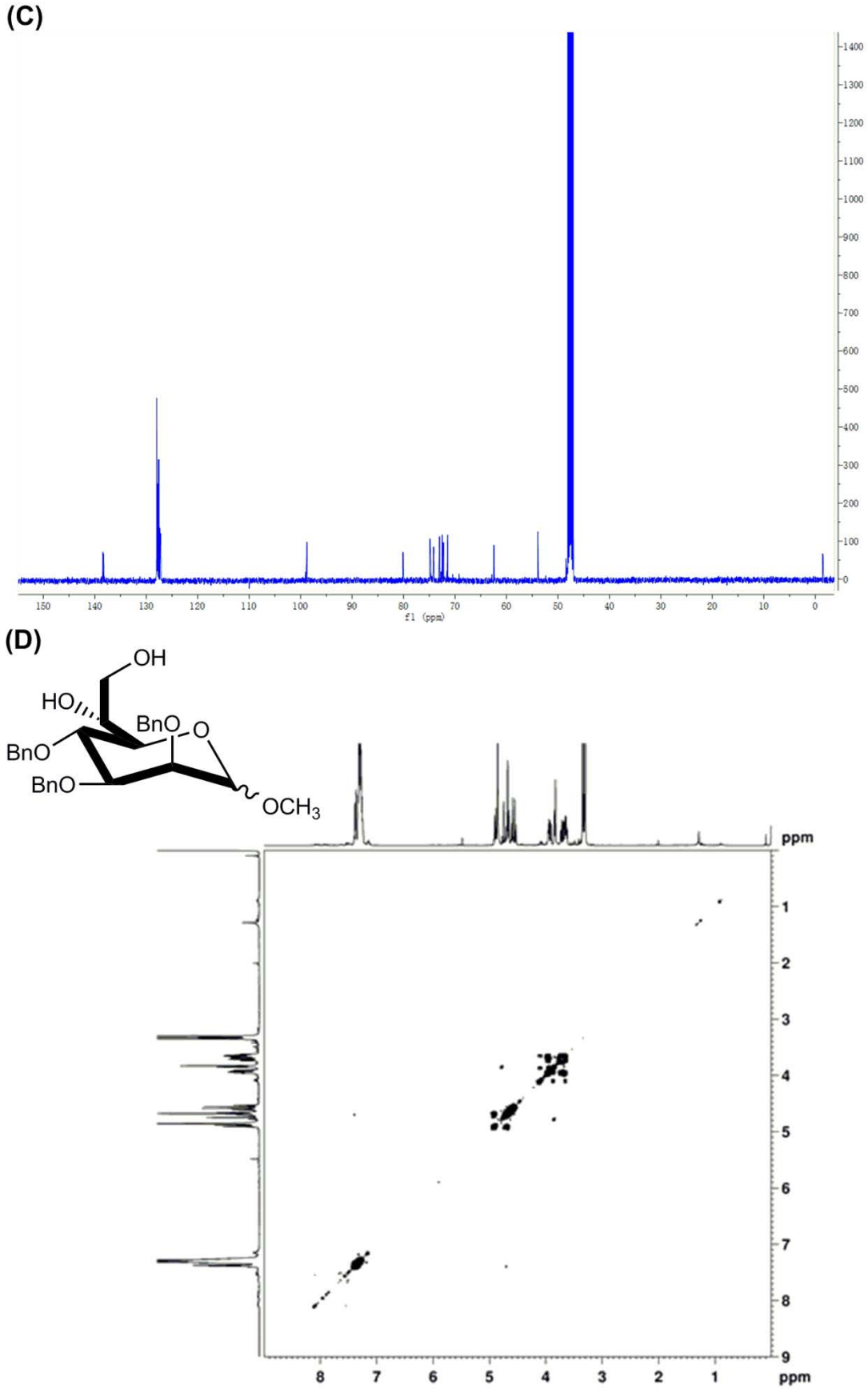

Figure S8-2. Spectral data of compound **1**. (**C**) ^{13}C NMR spectrum of compound **1**. (**D**) ^{1}H-^{1}H COSY spectrum of compound **1**.

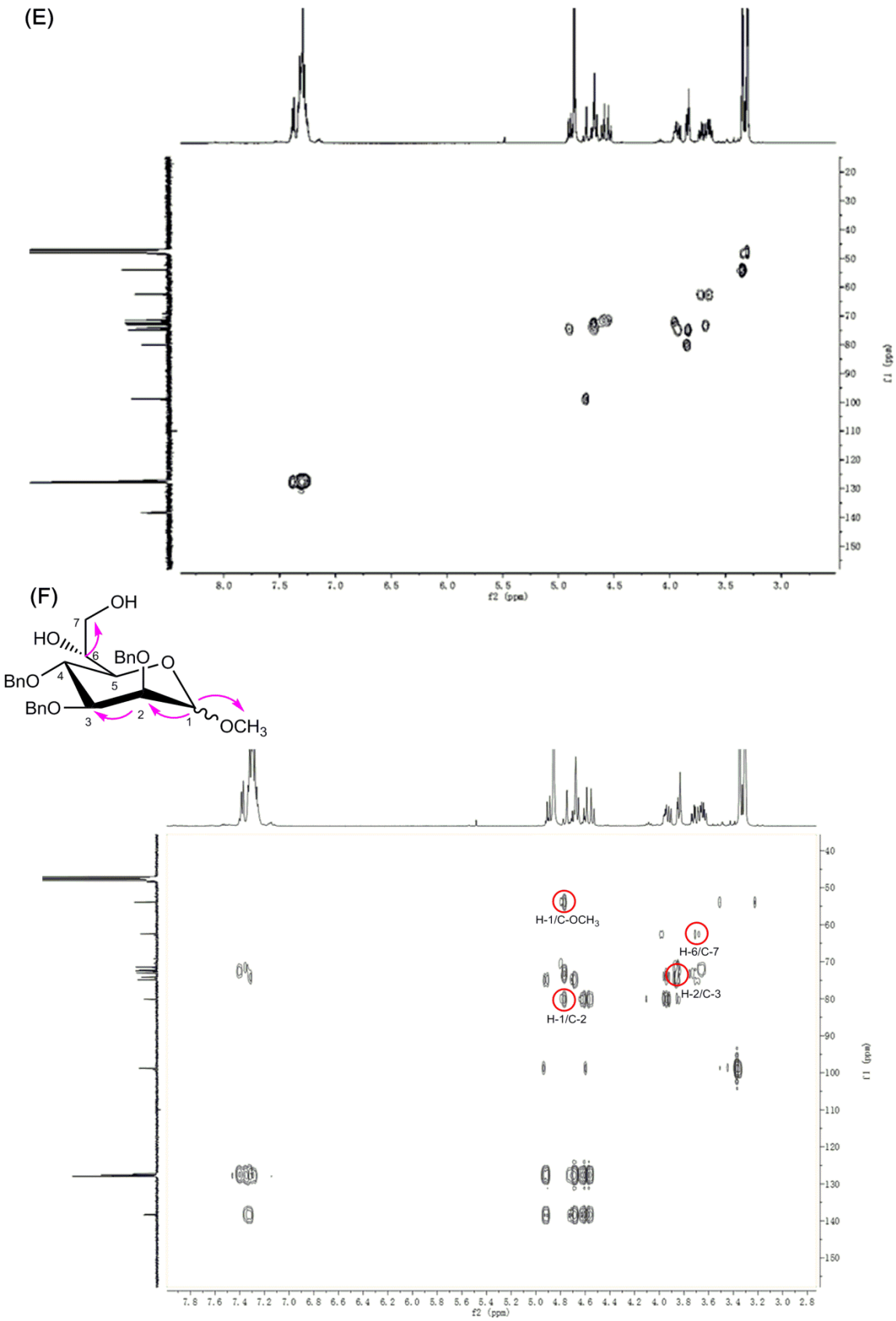

Figure S8-3. Spectral data of compound **1**. (F) HSQC spectrum of compound **1**. (G) HMBC spectrum of compound **1**.

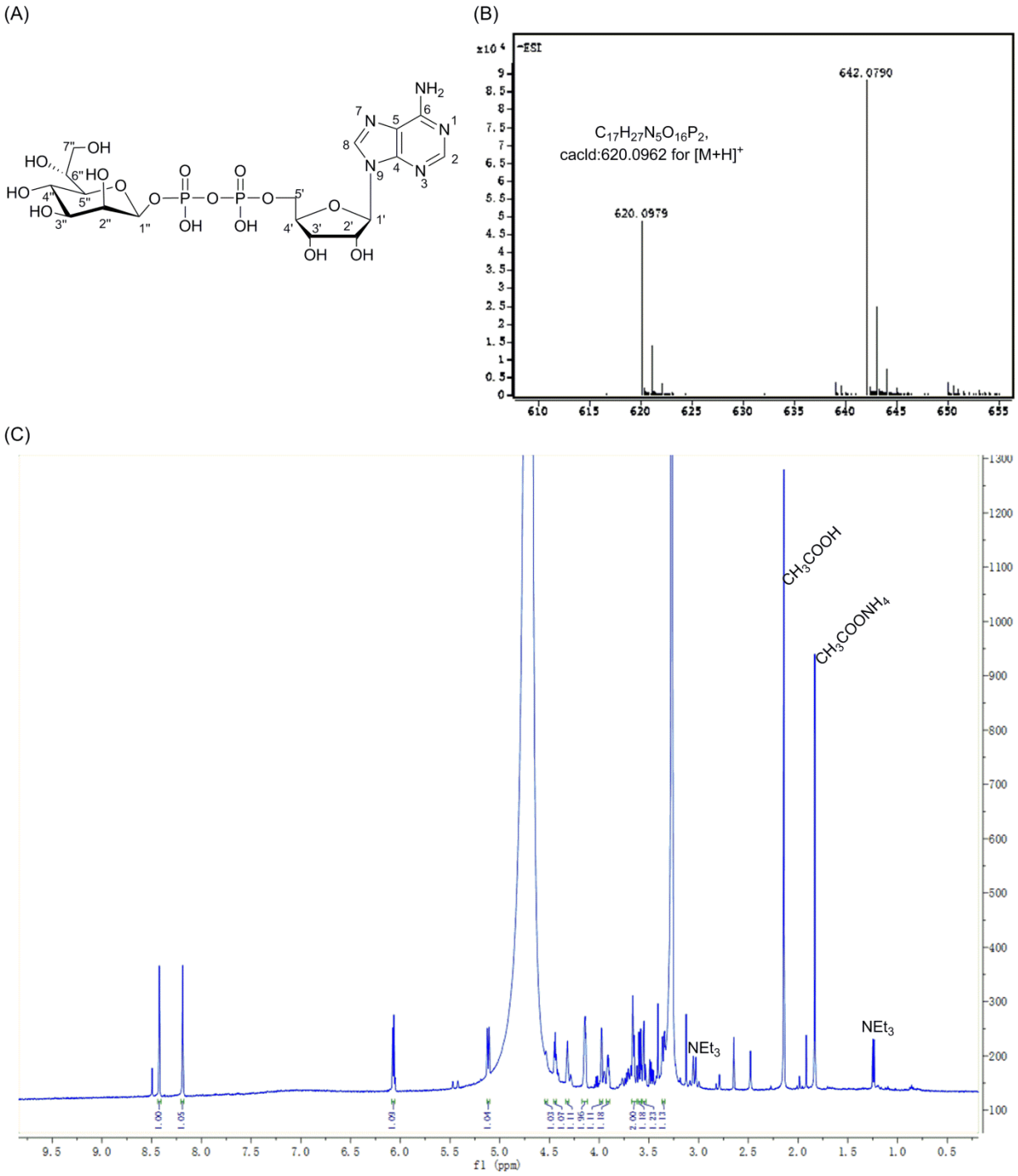

Figure S9. Spectral data of compound ADP-D-*glycero*-*β*-D-*manno*-heptose. (A) Structure of ADP-D-*glycero*-*β*-D-*manno*-heptose. (B) HR-ESI-MS spectrum of ADP-D-*glycero*-*β*-D-*manno*-heptose. (C) ^{1}H NMR spectrum of ADP-D-*glycero*-*β*-D-*manno*-heptose in D_2O.

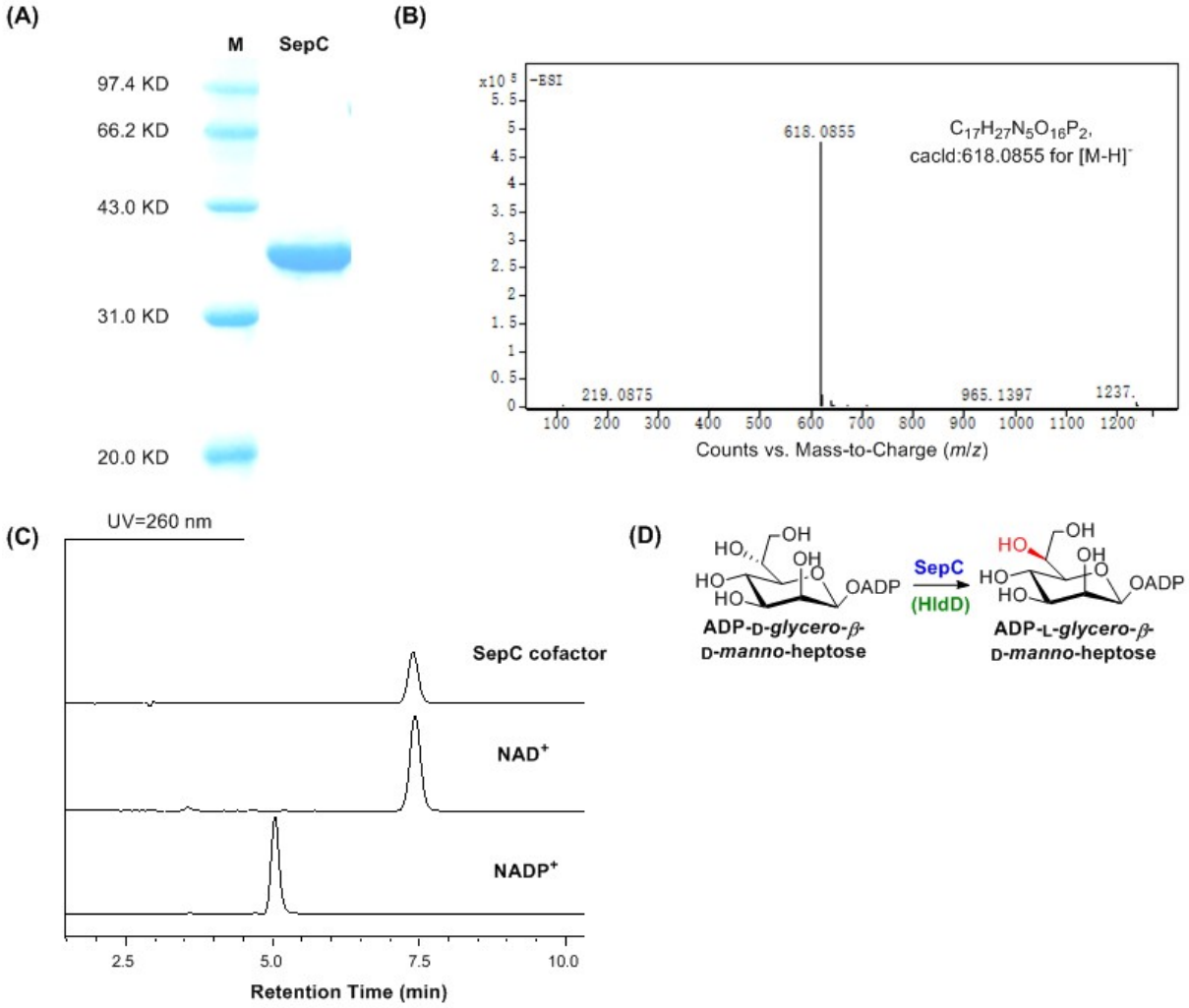

Figure S10. *In vitro* characterization of SepC. **(A)** SDS-PAGE analysis of SepC. Lane M, protein marker. **(B)** HR-ESI-MS spectrum of ADP-L-*glycero*-*β*-D-*manno*-heptose generated by the SepC assay. (C) HPLC analysis to determine the cofactor of SepC. (D) Reaction catalyzed by SepC. HldD, an isozyme of SepC from Gram-negative bacteria LPS heptose biosynthesis, is bracketed and indicated with green.

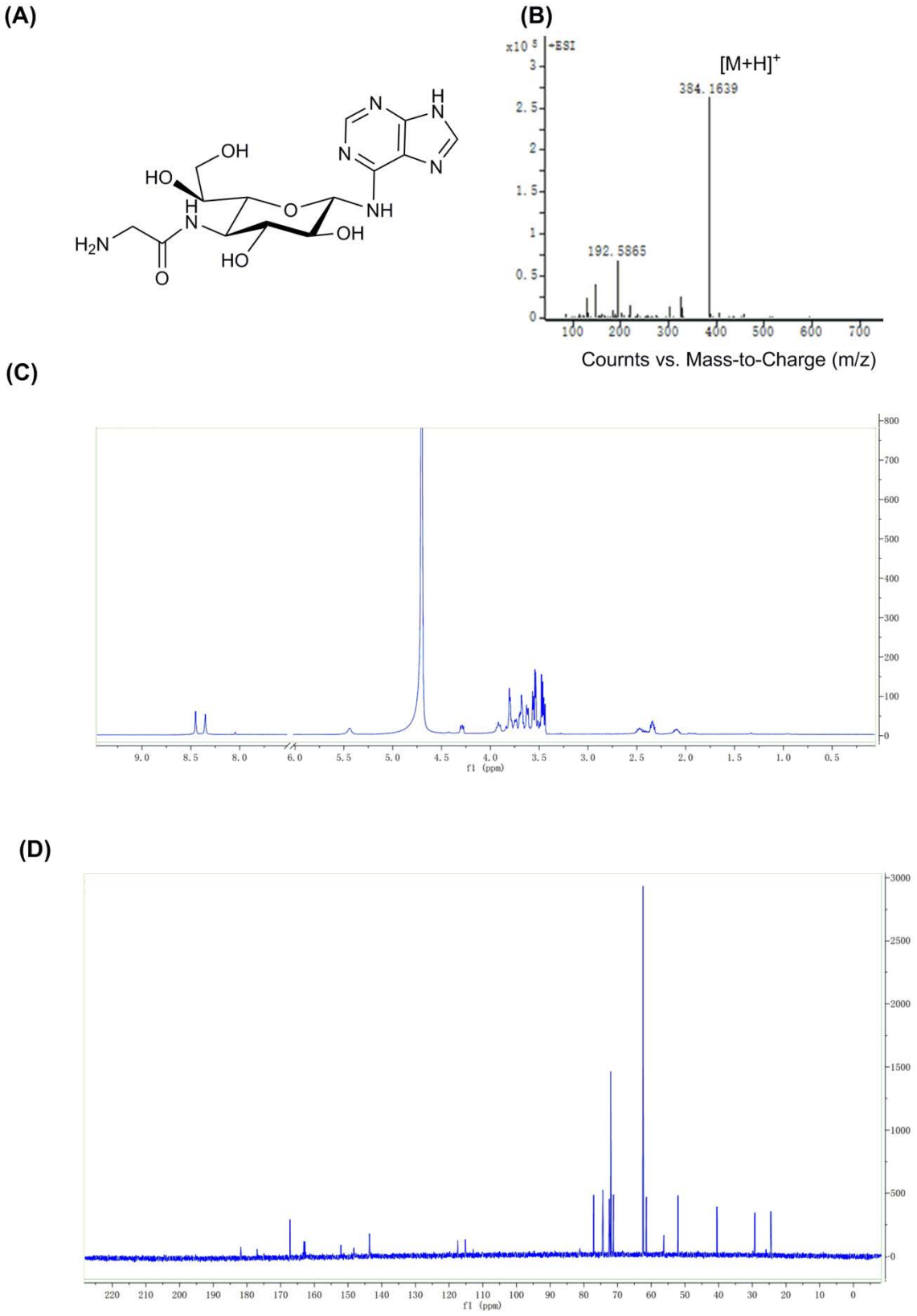

Figure S11. Spectral data of compound SEP-384. **(A)** Structure of compound SEP-384. **(B)** HR-ESI-MS spectrum of compound SEP-384. **(C)** ^{1}H NMR spectrum of compound SEP-384 in D_2O. **(D)** ^{13}C NMR spectrum of compound SEP-384 in D_2O.

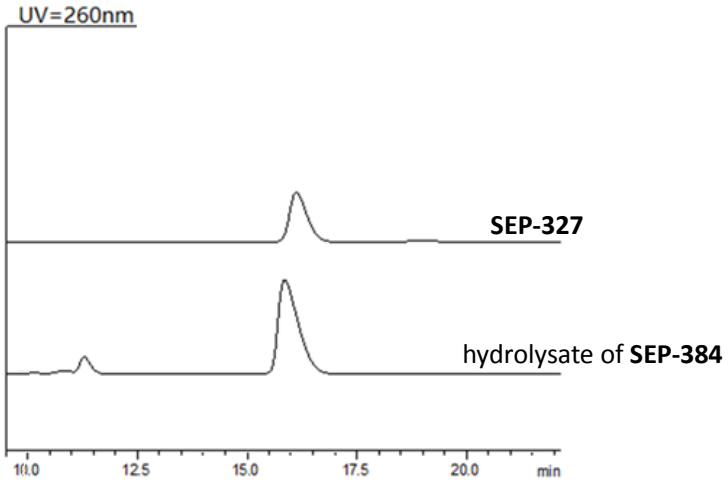

Figure S12. HPLC analysis of SEP-327 and the hydrolysate of SEP-384**.** The samples (SEP-327 and hydrolysate of SEP-384) were analyzed by analytic HPLC (Zorbax SB-Aq, 5 μm, 4.6 mm × 250 mm, Agilent, Santa Clara, CA, USA) eluted with water containing 0.1% trifluoroacetic acid at a flow rate of 1 mL/min with the detection UV wavelength at 260 nm. The retention time of hydrolysate of SEP-384 was 16.8 min, which is consistent with that of the authoritative SEP-327. The hydrolysate of SEP-384 was prepared by a modified methanolysis process. Briefly, the methanolysis of 1.0 mg SEP-384 was conducted for 2 hours in 1 mL MeOH with 1 M HCl at 50 ^{o}C. After extracted with chloroform, the aqueous layer was obtained as the hydrolysate of SEP-384.

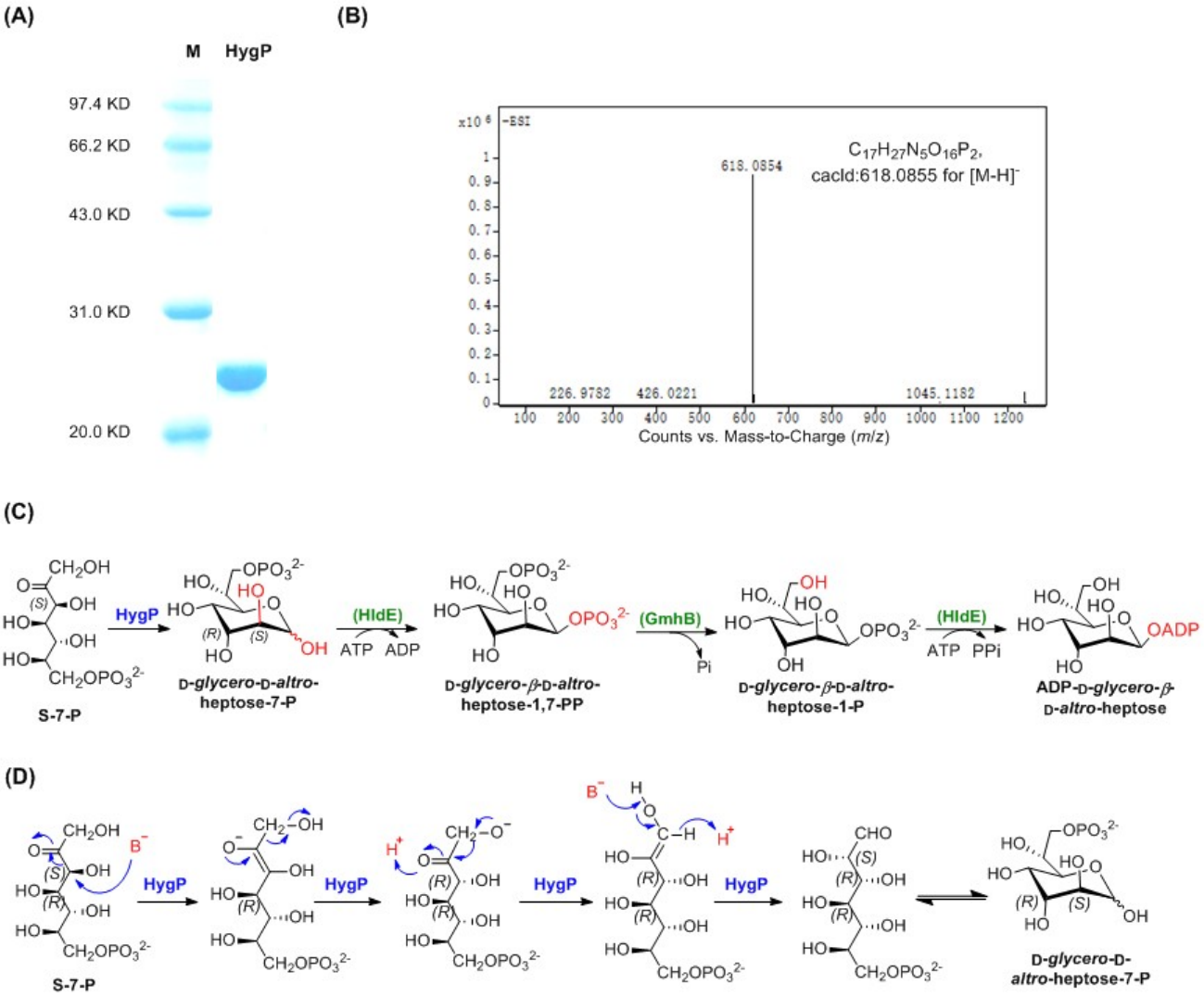

Figure S13. *In vitro* characterization of HygP with the hybrid HygP + GmhB + HldE assay. **(A)** SDS-PAGE analysis of HygP. Lane M, protein marker. **(B)** HR-ESI-MS spectrum of ADP-D-*glycero*-*β*-D-*altro*-heptose generated by the hybrid assay. **(C)** ADP-D-*glycero*-*β*-D-*altro*-heptose biosynthetic pathway catalyzed by HygP, GmhB and HldE. GmhB and HldE, the two enzymes from Gram-negative bacteria LPS heptose biosynthesis, are bracketed and indicated with green. **(D)** A proposed mechanism of the isomerization reaction catalyzed by HygP.

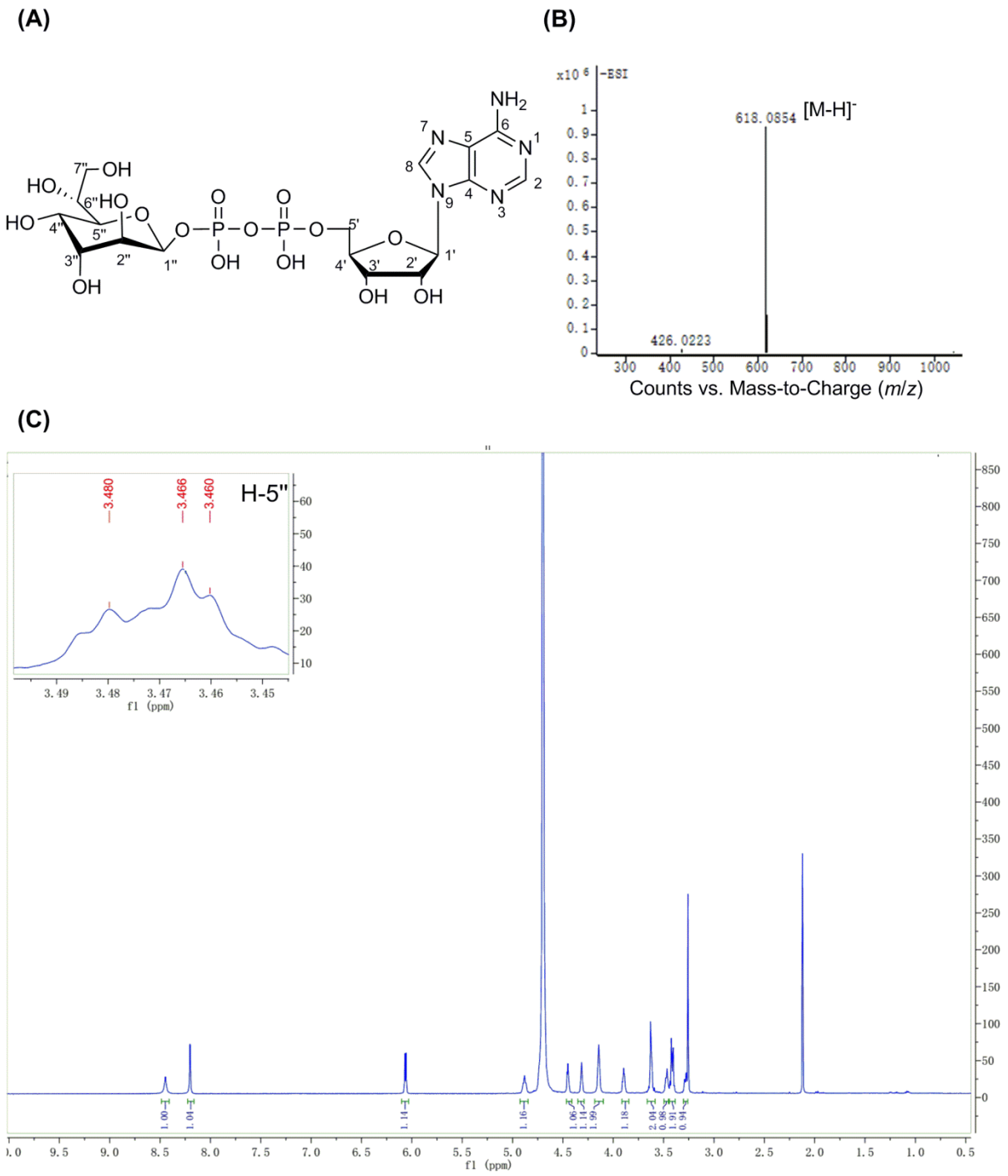

Figure S14-1. Spectral data of ADP-D-*glycero*-*β*-D-*altro*-heptose. **(A)** Structure of ADP-D-*glycero*-*β*-D-*altro*-heptose **(B)** HR-ESI-MS spectrum of ADP-D-*glycero*-*β*-D-*altro*-heptose. **(C)** ^{1}H NMR spectrum of refined ADP-D-*glycero*-*β*-D-*altro*-heptose in D_2O.

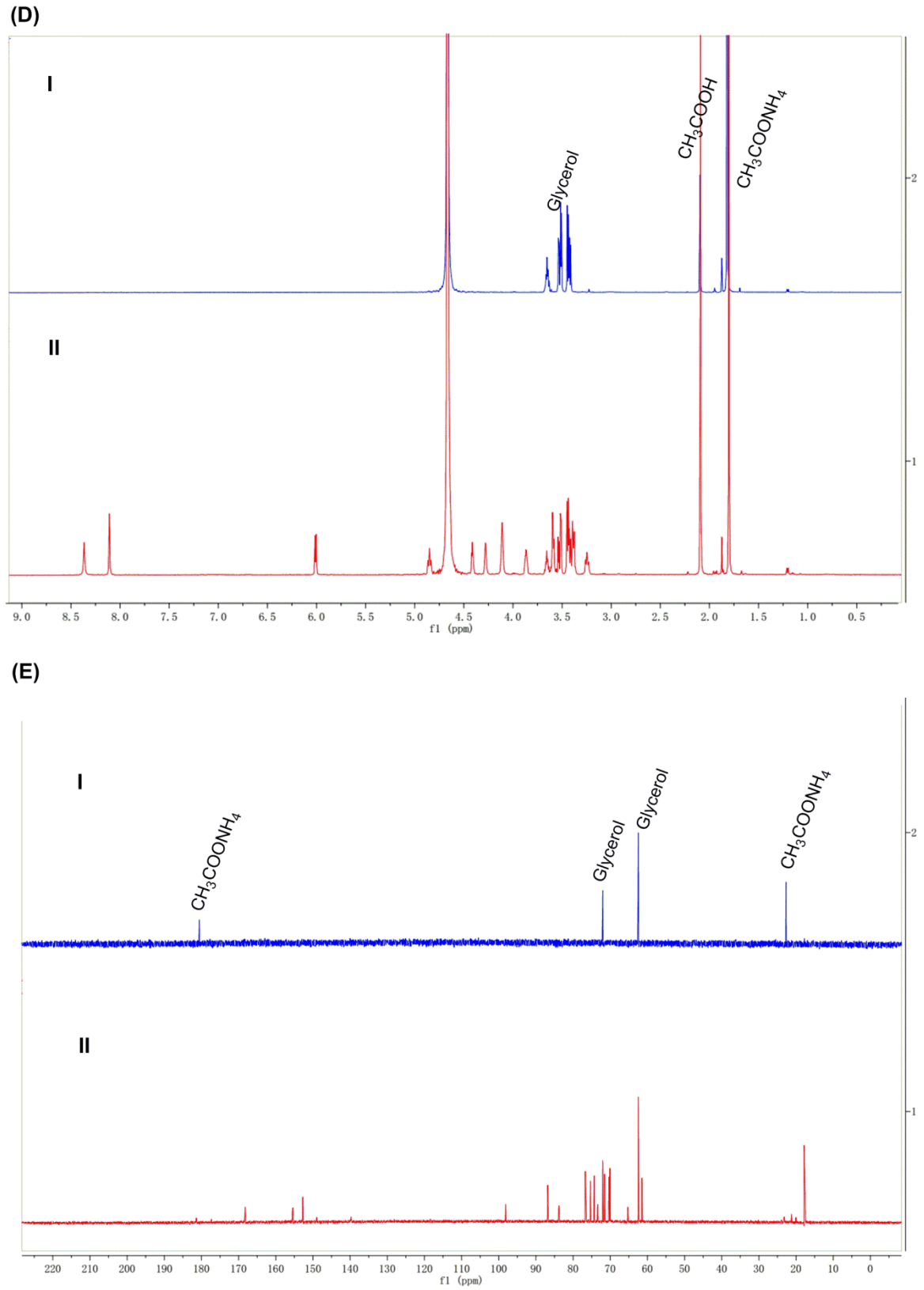

Figure S14-2. Spectral data of ADP-D-*glycero*-*β*-D-*altro*-heptose. **(D)** ^{1}H NMR spectrum of ADP-D-*glycero*-*β*-D-*altro*-heptose in D_2O. The upper is a ^{1}H NMR spectrum of the impurities, glycerol and CH_3COONH_4. **(E)** ^{13}C NMR spectrum of ADP-D-*glycero*-*β*-D-*altro*-heptose. The upper is a ^{13}C NMR spectrum of the impurities, glycerol and CH_3COONH_4.

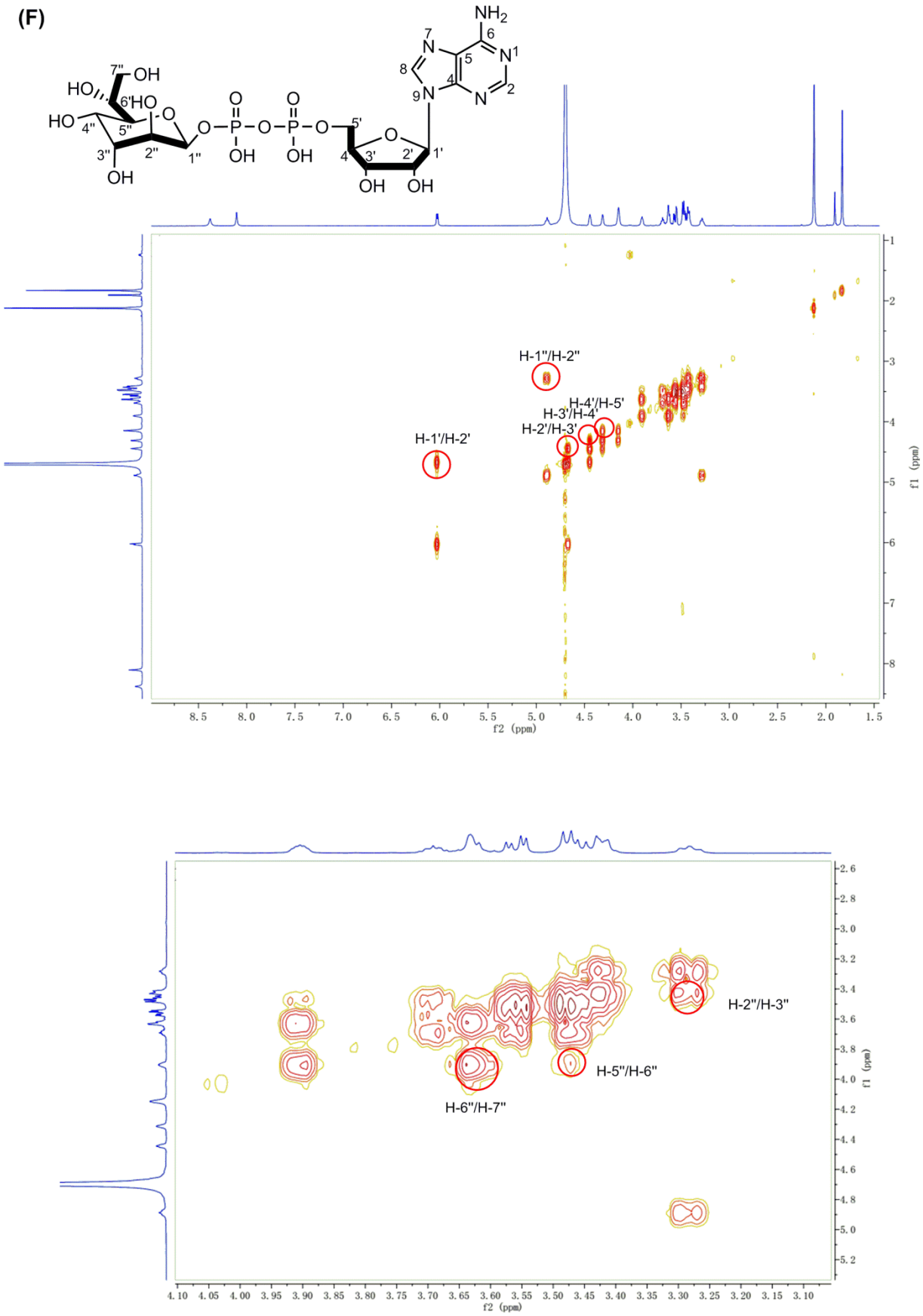

Figure S14-3. Spectral data of ADP-D-*glycero*-*β*-D-*altro*-heptose. **(F)** ^{1}H-^{1}H COSY spectrum of ADP-D-*glycero*-*β*-D-*altro*-heptose.

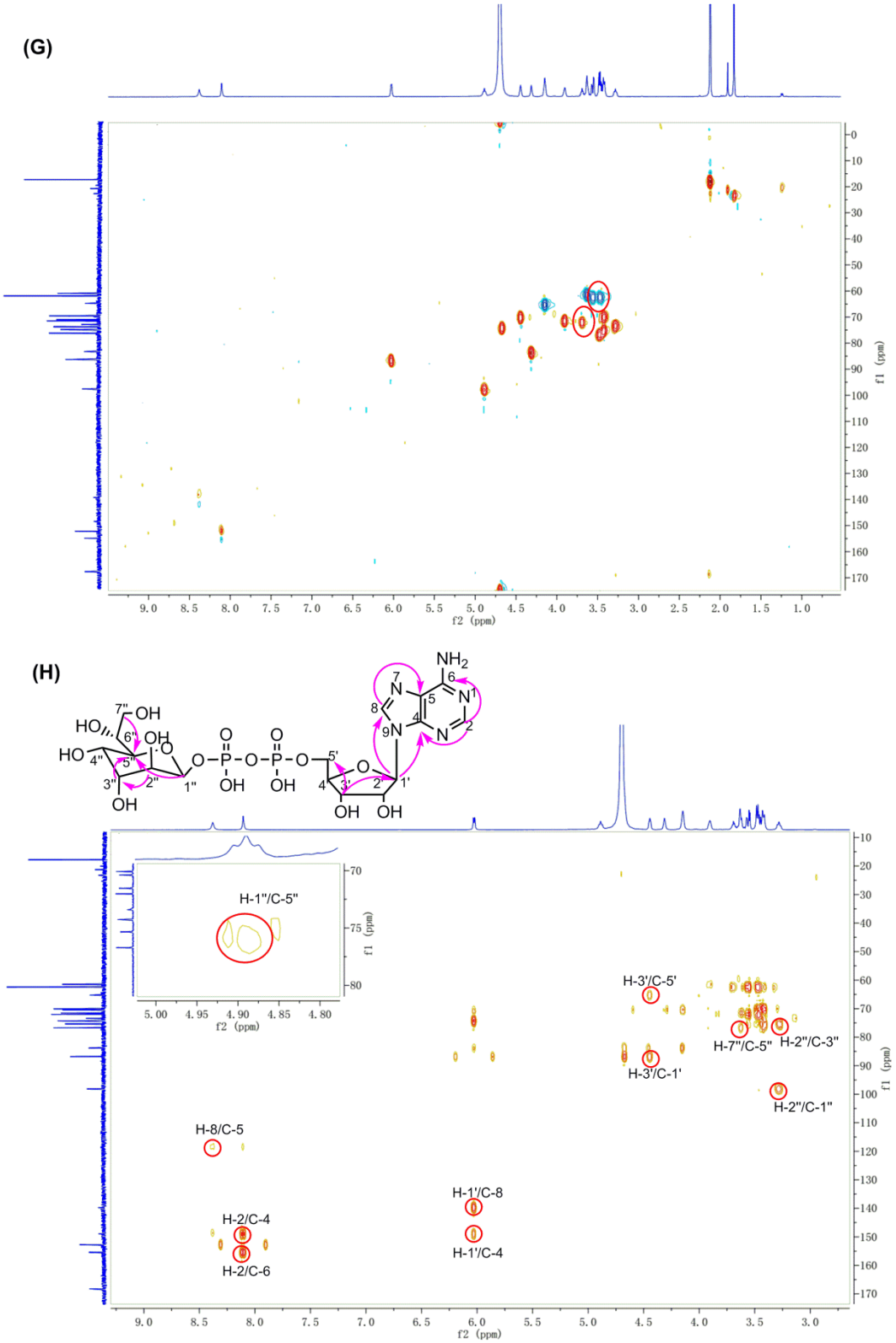

Figure S14-4. Spectral data of ADP-D-*glycero*-*β*-D-*altro*-heptose. **(G)** HMQC spectrum of ADP-D-*glycero*-*β*-D-*altro*-heptose**.** The signals of glycerol are marked with red cycles. **(H)** HMBC spectrum of ADP-D-*glycero*-*β*-D-*altro*-heptose. The key HMBC correlations are marked on the structure of ADP-D-*glycero*-*β*-D-*altro*-heptose with arrows and at the spectrum with red circles.

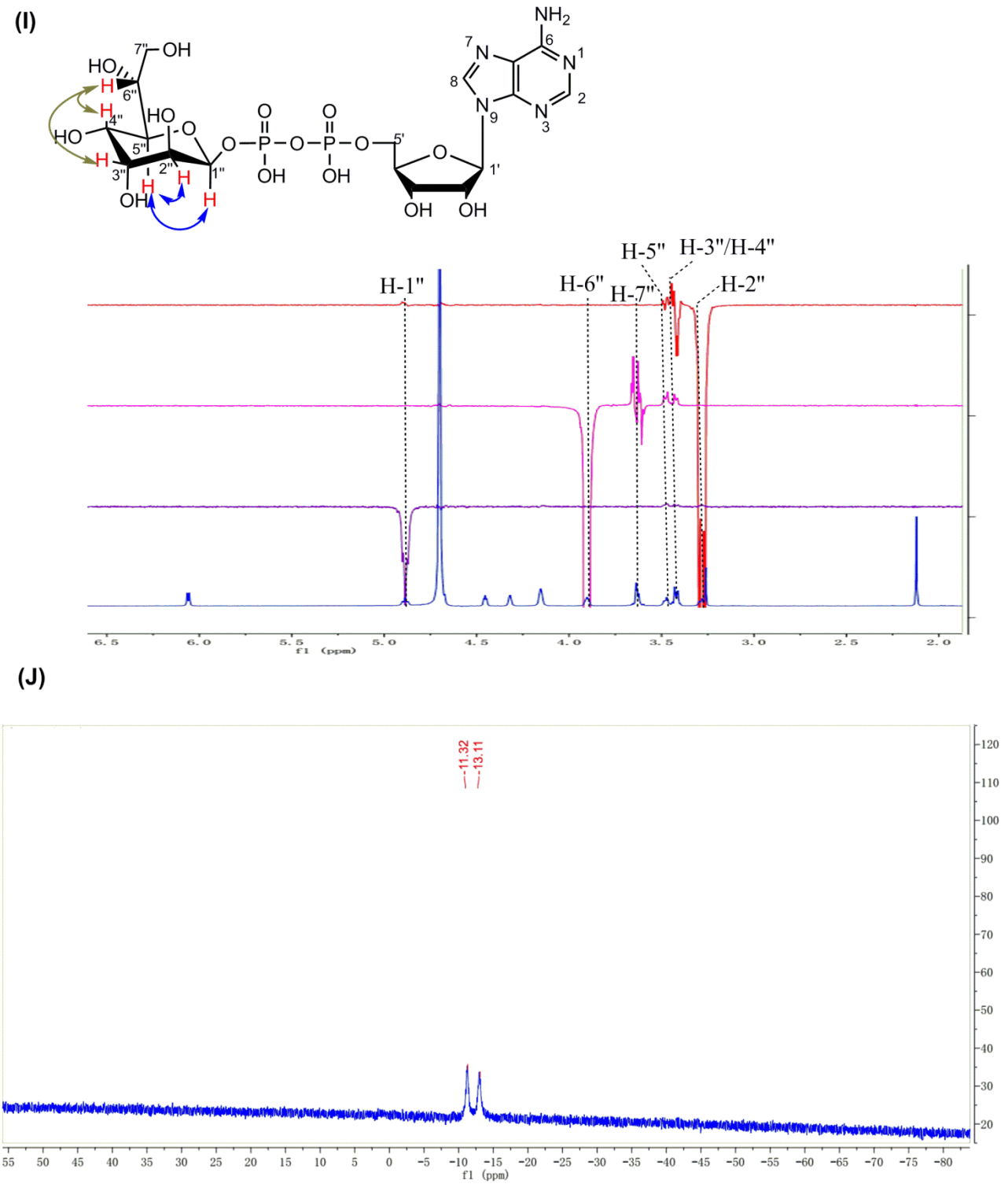

Figure S14-5. Spectral data of ADP-D-*glycero*-*β*-D-*altro*-heptose. **(I)** NOE difference spectrum of ADP-D-*glycero*-*β*-D-*altro*-heptose**.** The key NOE correlations are marked on the structure of ADP-D-*glycero*-*β*-D-*altro*-heptose with blue arrows. Since the signals of H-3″ and H-4″ are overlapping on ^{1}H NMR spectrum, the NOE correlations marked with gray arrows indicate that H-6″ has correlations with H-3″ and/or H-4″. **(J)** ^{31}P NMR spectrum of ADP-D-*glycero*-*β*-D-*altro*-heptose.