

## Supplementary Materials

### Origin of the 6/5/6/5 Tetracyclic Cyclopiazonic Acids

Wenyuan Zhang <sup>1</sup>, Xuejian Jiang <sup>1</sup>, Minjun Wang <sup>1</sup>, Zhizhen Zhang <sup>1</sup> and Nan Wang <sup>1,2,\*</sup>

<sup>1</sup> Ocean College, Zhejiang University, Zhoushan 316021, China; n\_wang@zju.edu.cn

<sup>2</sup> Hainan Institute of Zhejiang University, Sanya 572025, China; n\_wang@zju.edu.cn

\* Correspondence: n\_wang@zju.edu.cn (N.W.)

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Table S1. Strains, plasmids and mutants in this study.

| Strain / Plasmid         | Genotype/Function   | Source              |
|--------------------------|---|---------------------|
| <i>A. oryzae</i> HMP-F28 | Wild type strain  | In-house collection |
| <i>cpaA-R*::ptrA</i>     | HMP-F28 derived CpaA-R* domain deletion mutant                    | This study          |
| <i>cpaA-R*::acvA-TE</i>  | HMP-F28 derived CpaA-R* domain swapped with AcvA-TE domain mutant | This study          |
| <i>E. coli</i> DH5a      | Host for general cloning  | Takara              |
| pUC 57-sgRNA             | sgRNA carrier   | GensScript          |
| pUC 57-TE (AcvA)         | TE (AcvA) domain carrier  | GenScript           |
| pUC 57-SrfD              | TEII (SrfD) carrier   | Genewiz             |
| pPTR II                  | <i>ptrA</i> carrier   | In-house collection |
| pLD10001                 | Expression of PhoN and IpK  | This study          |
| pET28a-fgaPT2            | Expression of FgaPT2  | This study          |

Table S2. Primers used in this study.

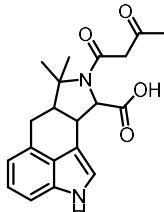
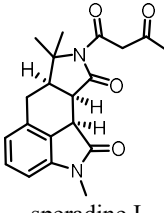
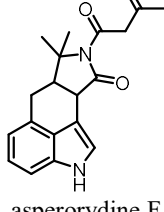
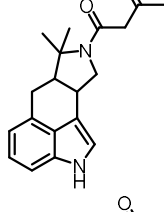
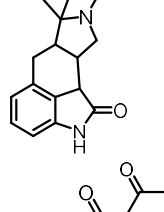
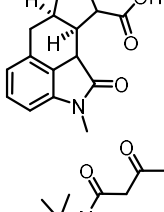
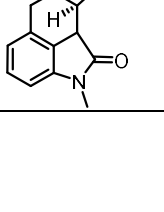
| Primer              | Nucleotide Sequence (5'-3')                                   | Function                                      |
|---------------------|---|---|
| R*KO/Swap_up_F      | AGAACTGTCCTGTGGTGCAGGACTCCTATGGTGTGACCGAGATTTC                | PCR targeting for R* domain knock-out or swap |
| R*KO_up_R           | agaatagcccagatagagttgagTTATTGCGACTGGAGGGCAAATTCG              | PCR targeting for R* domain knock-out         |
| R*Swap_up_R         | ctggcggcaataggaacaagatcggACAACCTGATCC TTGGCAATG               | PCR targeting for R* domain swap              |
| R*Swap_AcvA_F       | agcattgccaaaggatcaggtgtCCGATCTTGTTCCTA TTGC                   | PCR targeting for R* domain swap              |
| R*Swap_AcvA_R       | agaatagcccagatagagttgagCTAGAGAAATTTCTCGATTGTTGAACAAACGTG      | PCR targeting for R* domain swap              |
| R*KO_ptrA_F         | atttgcctccagtcgcaataaCTCAACTCTATCTCGG GCTATTCTTTTGATTATAAG    | PCR targeting for R* domain knock-out         |
| R*Swap_ptrA_F       | ttaacaatcgagaaattctctagCTCAACTCTATCTC GGGCTATTCTTTTGATTATAAGG | PCR targeting for R* domain swap              |
| R*KO/Swap_ptrA_R    | gaataagtggctcaataccctgAATAAGGGCGACACG GAAATGTTGAATAC          | PCR targeting for R* domain knock-out or swap |
| R*KO_down_F         | tcaacattccgtgtcgcccttattCAGGGTATTGAGCC ACTTATTCCGAGTTTC       | PCR targeting for R* domain knock-out         |
| R*Swap_down_F       | tcaacattccgtgtcgcccttattGGCAACAAGATACT CTTAACAC               | PCR targeting for R* domain swap              |
| R*KO/Swap_down_R    | AGATCTCCATTGCTCAGCTTTCGGGTGCC TGG                             | PCR targeting for R* domain knock-out or swap |
| R*KO_ID_up_ptrA_F   | CTGAATCACCATCAATGACAATCAACTG                                  | PCR confirmation                              |
| R*KO_ID_up_ptrA_R   | AATGAACCTCGTTAGCTCGTAATCCAC                                   | PCR confirmation                              |
| R*KO_ID_down_ptrA_F | CTGAGATAGAGAATTGTGTGGGATGAG                                   | PCR confirmation                              |
| R*KO_ID_down_ptrA_R | TACCCTGAATCAGAACTGCATAAGC                                     | PCR confirmation                              |

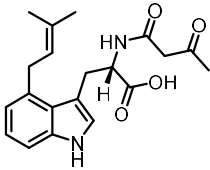
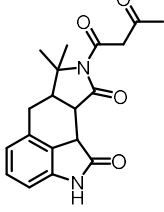
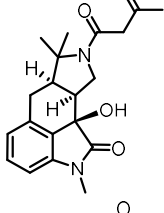
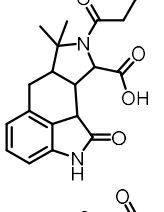
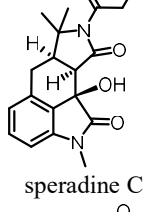
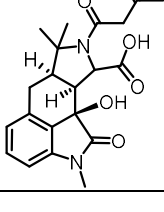
|                           |   |  |
|---------------------------|---|--|
| R*Swap_ID_acvA-<br>ptrA_F | ATCCCAACCAGAACCAGCCGATTTCC                            | PCR confirmation   |
| R*Swap_ID_acvA-<br>ptrA_R | GTGCCATGGTTCTCAGTGGTGTCAAGG                           | PCR confirmation   |
| R*Swap_ID_up-<br>acvA_F   | GGTCAGATTACAAGGGGCGATC                                | PCR confirmation   |
| R*Swap_ID_up-<br>acvA_R   | AACTTGTCGTTACCCCTAGTAGC                               | PCR confirmation   |
| R*Swap_ID_ptrA-<br>down_F | AGGAGGGGTTGAGTTAAATAGCATG                             | PCR confirmation   |
| R*Swap_ID_ptrA-<br>down_R | GATAACGCATTCTGAGATGTAAACACAG                          | PCR confirmation   |
| srfDExp_up_F              | TATATGCTGCCACTGGTGGTTCTCCACTTG                        | PCR targeting for <i>srfD</i><br>expression                |
| srfDExp_up_R              | taaaatgatcaaaacaccatGTTGGCGATGGAGGGG<br>TAG           | PCR targeting for <i>srfD</i><br>expression                |
| srfDExp_Pro_amy<br>B_F    | gctaccctccatgccaacATGGTGTTTTGATCATT<br>TTAAATTTTATATG | PCR targeting for <i>srfD</i><br>expression                |
| srfDExp_Pro_amy<br>B_R    | gatttaaagagctggaccatTGTGGGGTTTATTGTTC<br>AGAGAAGG     | PCR targeting for <i>srfD</i><br>expression                |
| srfDExp_srfD_F            | tctctgaacaataaaccccaATGGTCCAGCTCTTT<br>AAATC          | PCR targeting for <i>srfD</i><br>expression                |
| srfDExp_srfD_R            | cttcacgagctactacagatcCTATAACAATTGGCCT<br>GC           | PCR targeting for <i>srfD</i><br>expression                |
| srfDExp_Tmn_ami<br>yB_F   | ctgcaggccaattgttatagGATCTGTAGTAGCTCGT<br>GAAG         | PCR targeting for <i>srfD</i><br>expression                |
| srfDExp_Tmn_ami<br>yB_R   | gagtacgtatccaccactacGGATCCTTTCCTATAAT<br>AGAC         | PCR targeting for <i>srfD</i><br>expression                |
| srfDExp_down_F            | tctattataggaaaggatccGTAGTGGTGGATACGTA<br>CTCCTTTTATG  | PCR targeting for <i>srfD</i><br>expression                |
| srfDExp_down_R            | GTTGCGGATCTTGCTGCTTGCGGAG                             | PCR targeting for <i>srfD</i><br>expression                |
| pET28aFgaPT2N_<br>His_F   | GGAATTCCATATGATGAAGGCAGCCAATG<br>CCTC                 | PCR targeting for <i>fgaPT2</i><br>expression with his tag |
| pET28aFgaPT2N_<br>His_R   | CCCAAGCTTTCAGTGGAGACCGGAATAAT<br>ATACC                | PCR targeting for <i>fgaPT2</i><br>expression with his tag |

Table S3. TE domains of different origins.

| NRPS/PKS | Structure              | Source                             | Reference |
|----------|------------------------|------------------------------------|-----------|
| AcvA     | A-T-C-A-T-C-A-T-E-TE   | <i>Aspergillus nidulans</i>        | [24]      |
| ApvA     | A-T-TE                 | <i>Aspergillus terreus</i>         | [34]      |
| BtyA     | A-T-TE                 | <i>Aspergillus terreus</i>         | [34]      |
| PngA     | A-T-TE                 | <i>Aspergillus terreus</i>         | [34]      |
| PesM     | A-T-C-A-T-C-E-A-T-C-TE | <i>Aspergillus fumigatus</i> AF293 | [35]      |
| SrfA-C   | C-A-PCP-TE             | <i>Bacillus subtilis</i>           | [36]      |
| TMC      | KS-AT-KR-ACP-TE        | <i>Streptomyces</i> sp. CK4412     | [37]      |

Table S4. Hypothetic E-ring opening compounds subjected for EIC analysis.

| No. | Structure  | Calculated Mass  | EIC Found   |
|-----|--|--|---|
| 1   |  <p><i>seco-α-CPA</i></p> | $[M+H]^+$ , $m/z$ 355.1652,<br>$[M+Na]^+$ , $m/z$ 377.1472 | 355.1647/377.1477 (WT, $R_t$ 15~16 min);<br>355.1651/377.1455 (WT, $R_t$ 19~20 min);<br>355.1656/377.1471 (WT, $R_t$ 25~26 min) |
| 2   |  <p>speradine I</p>       | $[M+H]^+$ , $m/z$ 355.1652,<br>$[M+Na]^+$ , $m/z$ 377.1472 | 355.1647/377.1477 (WT, $R_t$ 15~16 min);<br>355.1651/377.1455 (WT, $R_t$ 19~20 min);<br>355.1656/377.1471 (WT, $R_t$ 25~26 min) |
| 3   |  <p>asperorydine F</p>   | $[M+H]^+$ , $m/z$ 325.1547,<br>$[M+Na]^+$ , $m/z$ 347.1366 | Not Detected  |
| 4   |                         | $[M+H]^+$ , $m/z$ 311.1754,<br>$[M+Na]^+$ , $m/z$ 333.1573 | Not Detected  |
| 5   |                         | $[M+H]^+$ , $m/z$ 327.1703,<br>$[M+Na]^+$ , $m/z$ 349.1523 | Not Detected  |
| 6   |                         | $[M+H]^+$ , $m/z$ 385.1758,<br>$[M+Na]^+$ , $m/z$ 407.1577 | 385.1774/ 407.1575 (WT, $R_t$ 12~13 min);<br>385.1769/407.1572 ( <i>cpaA-R*::ptrA</i> + $\beta$ -CPA, $R_t$ 12~13 min)          |
| 7   |                         | $[M+H]^+$ , $m/z$ 341.1860,<br>$[M+Na]^+$ , $m/z$ 363.1679 | Not Detected  |

|    |  |   |  |
|----|--|---|--|
| 8  |  <p>PAATrp</p>        | <p><math>[M+H]^+</math>, <math>m/z</math> 357.1809,<br/> <math>[M+Na]^+</math>, <math>m/z</math> 379.1628</p> | <p>357.1801/379.1622 (<i>cpaA-R*::ptrA</i> +<br/> PAATrp, <math>R_t</math> 18~19 min)</p>  |
| 9  |                       | <p><math>[M+H]^+</math>, <math>m/z</math> 341.1496,<br/> <math>[M+Na]^+</math>, <math>m/z</math> 363.1315</p> | <p>Not Detected</p>  |
| 10 |                       | <p><math>[M+H]^+</math>, <math>m/z</math> 357.1809,<br/> <math>[M+Na]^+</math>, <math>m/z</math> 379.1628</p> | <p>Not Detected</p>  |
| 11 |                      | <p><math>[M+H]^+</math>, <math>m/z</math> 371.1601,<br/> <math>[M+Na]^+</math>, <math>m/z</math> 393.1421</p> | <p>371.1553/393.1373 (<i>cpaA-R*::ptrA</i> + <math>\alpha</math>-<br/> CPA, <math>R_t</math> 15~16 min);<br/> 371.1606/393.1425 (<i>cpaA-R*::ptrA</i> + <math>\beta</math>-<br/> CPA, <math>R_t</math> 14~15 min);</p> |
| 12 |  <p>speradine C</p> | <p><math>[M+H]^+</math>, <math>m/z</math> 371.1601,<br/> <math>[M+Na]^+</math>, <math>m/z</math> 393.1421</p> | <p>371.1553/393.1373 (<i>cpaA-R*::ptrA</i> + <math>\alpha</math>-<br/> CPA, <math>R_t</math> 15~16 min);<br/> 371.1606/393.1425 (<i>cpaA-R*::ptrA</i> + <math>\beta</math>-<br/> CPA, <math>R_t</math> 14~15 min);</p> |
| 13 |                     | <p><math>[M+H]^+</math>, <math>m/z</math> 401.1707,<br/> <math>[M+Na]^+</math>, <math>m/z</math> 423.1527</p> | <p>Not Detected</p>  |

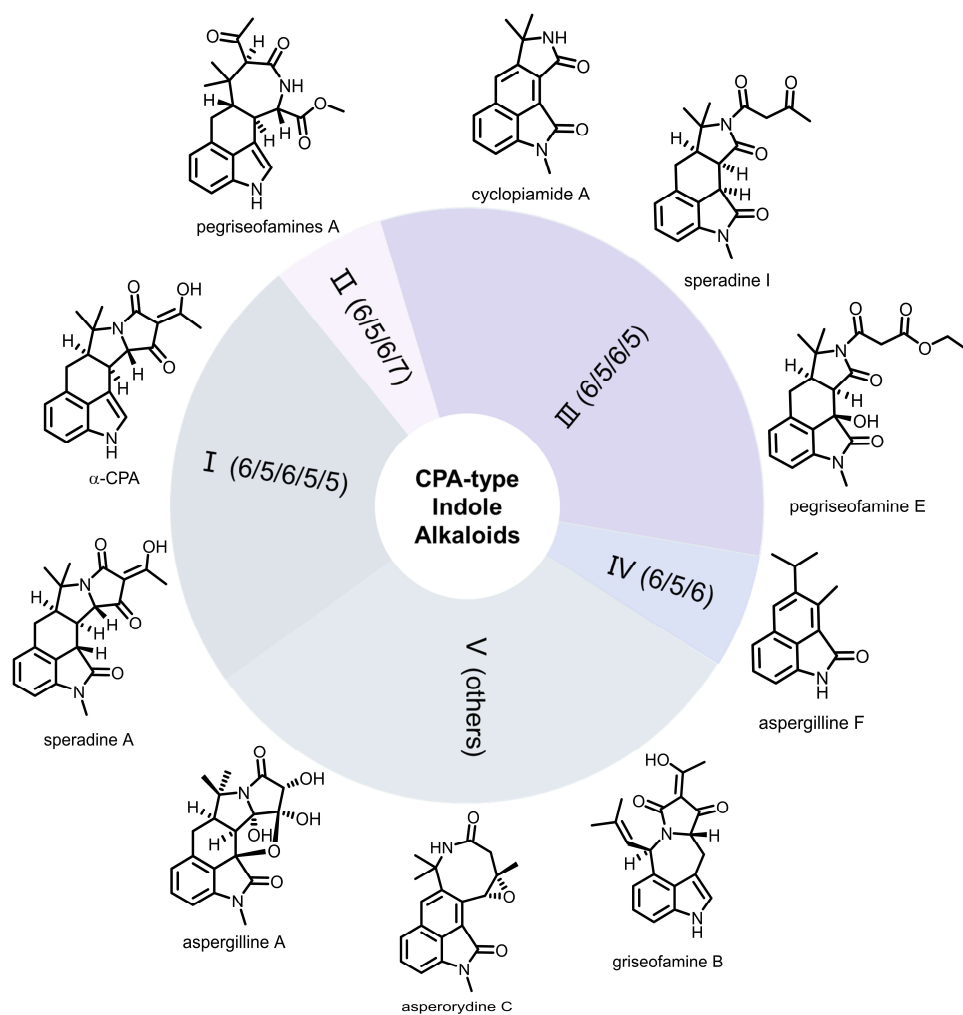


Figure S1. The reported skeletons of CPA-type compounds.

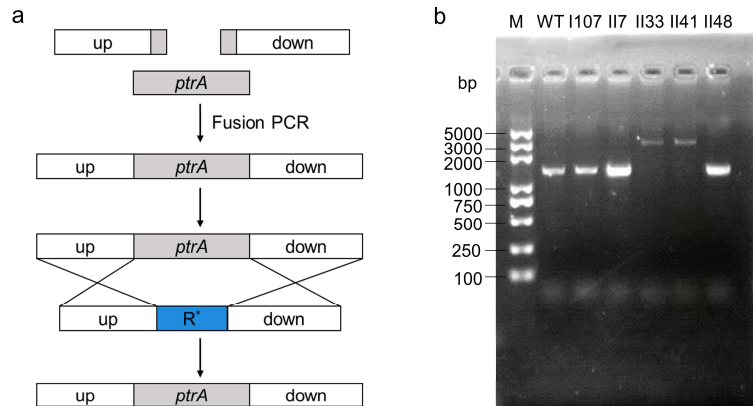


Figure S2. Construction (a) and genotype verification (b) of *cpaA-R\*::ptrA* mutants. a. the diagram for *cpaA-R\** gene knockout by homologous recombination, up and down: homology arm of *cpaA-R\**, *ptrA*: pyrithiamine; b. agarose gel electrophoresis of *cpaA-R\*::ptrA* mutants showed that II33 and II41 were identified to be the correct mutants, M: DNA marker.

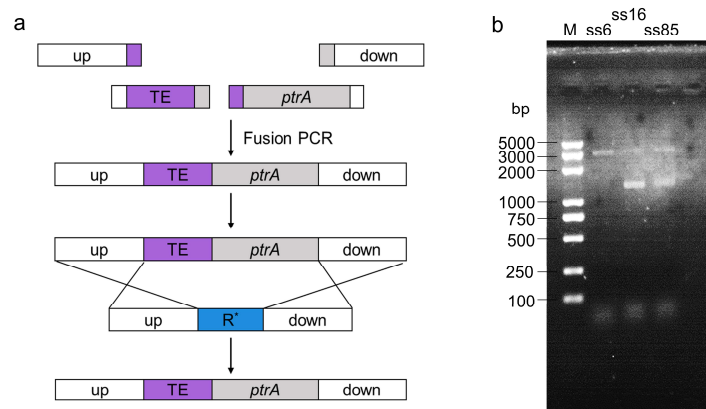


Figure S3. Construction (a) and genotype verification (b) of the domain swapped mutants (*cpaA-R\*::acvA-TE*). a. the diagram showing replacement of *cpaA-R\** with *acvA-TE* by homologous recombination, up and down: homology arm of *cpaA-R\**, *ptrA*: pyrithiamine, TE: AcvA-TE domain; b. Transformant ss6 was identified as a correct mutant by agarose gel electrophoresis, M: DNA marker.

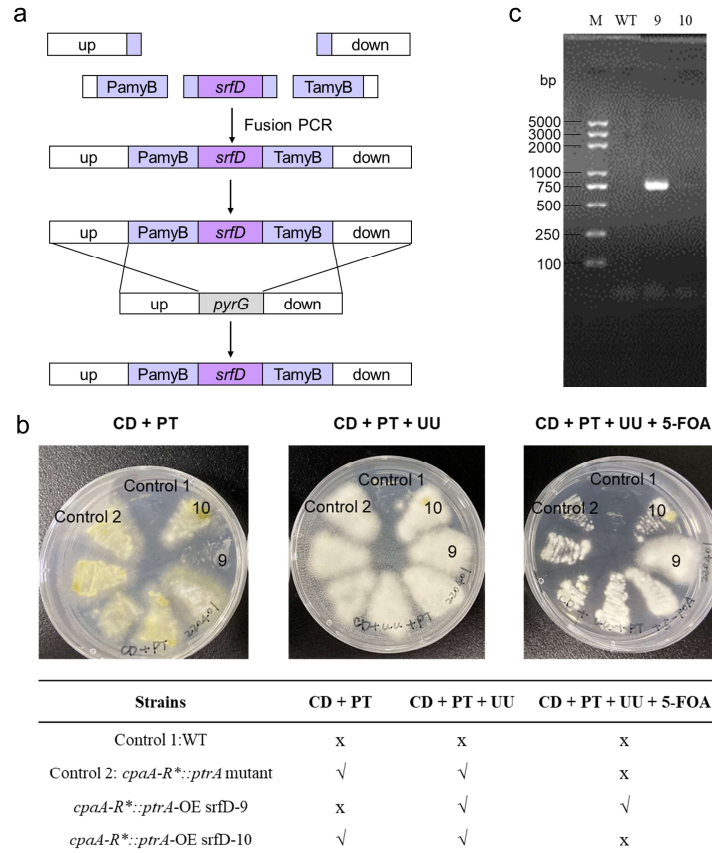


Figure S4. Construction (a), screening (b) and verification (c) of the TEII expressing mutant by introduce *srfD* gene encoding a TEII to a *cpaA-R\*::ptrA* mutant. a. construction of the *srfD* overexpressing DNA fragment for homologous recombination, up and down: homology arm of *pyrG*; *pyrG*: orotidine-5'-phosphate decarboxylase, *srfD*: thioesterase, PamyB: amyB promoter, TamyB: amyB terminator; b. growth phenotype of *cpaA-R\*::ptrA*-OE *srfD* mutants, WT strain (Control 1, wild type) couldn't growth on the CD+PT, CD+PT+UU and CD+PT+UU+5-FOA plates; the *cpaA-R\*::ptrA* mutants (Control 2) with the *ptrA* marker grew on CD+PT and CD+PT+UU plates, but could not grow on CD+PT+UU+5-FOA plate; the correct *cpaA-R\*::ptrA*-OE *srfD* mutant showed growth on the CD+PT+UU and CD+PT+UU+5-FOA plates, but not on the CD+PT plate without uridine and uracil; the *cpaA-R\*::ptrA*-OE *srfD*-9 was identified as a correct mutant. CD: Czapek-Dox medium; PT: pyrithiamine; UU: uridine and uracil; 5-FOA: 5-fluoroorotic acid. c. agarose gel electrophoresis of *cpaA-R\*::ptrA*-OE *srfD* mutants, further confirmed the genotype of *cpaA-R\*::ptrA*-OE *srfD* -9, M: DNA marker.



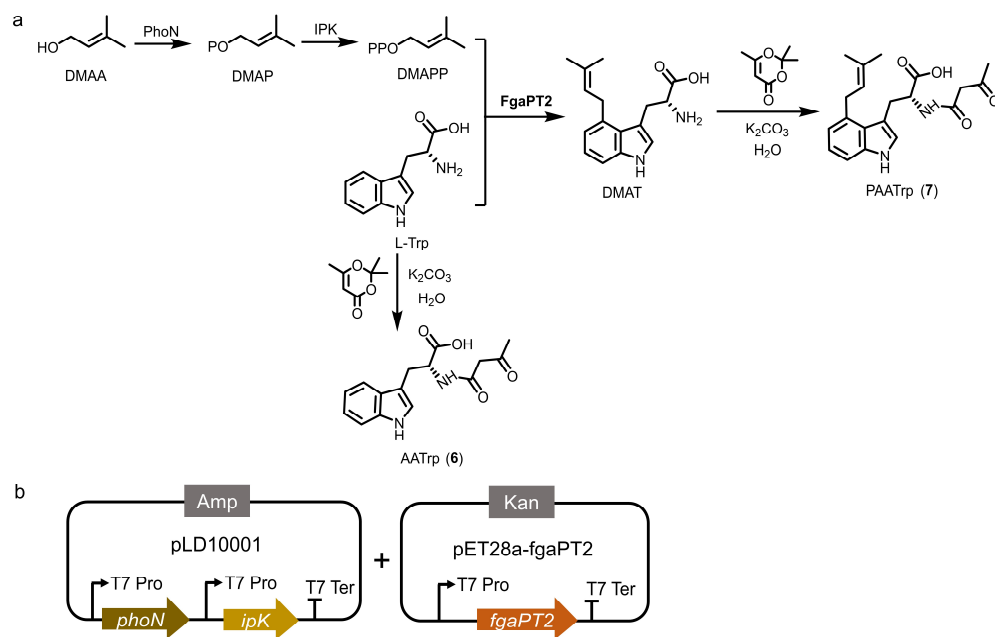


Figure S5. Synthesis of AATrp and PAATrp by whole cell biotransformation and chemical reactions (a). The plasmids used for biosynthesis of DMAPP (dimethylallyl diphosphate) (pLD10001) [34] and prenylation of L-tryptophan (pET28a-fgaPT2) in *E. coli* Rosetta (DE3) (b).

Table S5. NMR assignments for  $\beta$ -CPA,  $\alpha$ -CPA and PAATrp ( $^1\text{H}$ , 600 MHz,  $^{13}\text{C}$  150 MHz).

| $\beta$ -CPA |                                 | $\alpha$ -CPA   |                              | PAATrp          |   |                 |
|--------------|---------------------------------|-----------------|------------------------------|-----------------|---|-----------------|
| No.          | $^1\text{H}$                    | $^{13}\text{C}$ | $^1\text{H}$                 | $^{13}\text{C}$ | $^1\text{H}$  | $^{13}\text{C}$ |
| 1            |                                 |                 | 8.16 (1H br s)               |                 |   | 176             |
| 2            | 7.03 (1H, m)                    | 123.1           | 7.17 (1H s)                  | 123.0           | 4.72 (1H, dd, $J=9.6, 4.4$ Hz)                                      | 55.9            |
| 3            |                                 | 111.7           |                              | 110.0           | 3.22 (1H, dd, $J=15.1, 9.5$ Hz),<br>3.57 (1H, dd, $J=15.1, 4.6$ Hz) | 30.8            |
| 4            | 3.67 (2H, m), 2.85 (2H, m)      | 30.2            | 3.67 (1H dd, $J=11.0, 5.9$ ) | 26.5            |   | 111.8           |
| 5            | 4.08 (1H, dd, $J=11.1, 3.3$ Hz) | 63.6            | 4.18 (1H, d, $J=11.1$ )      | 71.8            |   | 126.4           |
| 6            |                                 | 194.5           |                              |                 |   | 135.2           |
| 7            |                                 | 101.8           |                              | 63.4            | 6.75 (1H, d, $J=7.1$ Hz)  | 120.4           |
| 8            |                                 | 174.7           |                              | 36.1            | 6.96 (1H, t, $J=7.6$ Hz)  | 122.4           |
| 9            |                                 |                 |                              | 26.4            | 7.15 (1H, d, $J=8.1$ Hz)  | 110.4           |
| 10           |                                 | 133.0           |                              | 128.7           |   | 138.8           |
| 11           | 5.33 (1H, m)                    | 123.4           | 2.64 (1H, m)                 | 116.5           | 7.08 (1H, s)  | 124.5           |
| 12           | 3.75 (2H, m)                    | 32.4            | 3.07 (2H, m)                 | 120.8           | 3.76 (2H, t, $J=5.7$ Hz)  | 33.4            |
| 13           |                                 | 134.8           |                              | 108.7           | 5.32 (1H, m)  | 125.6           |
| 14           | 6.93 (1H, d, $J=7.2$ Hz)        | 120.6           | 7.20 (1H, d, $J=8.0$ )       | 133.4           |   | 132.7           |
| 15           | 7.14 (1H, t, $J=7.6$ Hz)        | 122.9           | 7.14 (1H, m)                 | 125.9           | 1.76 (3H, s)  | 18.2            |
| 16           | 7.24 (1H, m, $J=8.2$ Hz)        | 109.6           | 6.92 (1H, d, $J=6.9$ )       | 195.2           | 1.74 (3H, s)  | 25.9            |
| 17           |                                 | 137.4           |                              | 105.6           |   | 168.8           |
| 18           |                                 | 124.8           |                              | 175.2           | 3.3 (2H)  | 49.6            |
| 19           |                                 | 185.6           |                              | 184.5           |   | 204.7           |
| 20           | 2.49 (3H, s)                    | 19.8            | 2.46 (3H, s)                 | 24.3            | 2.08 (3H, s)  | 29.8            |
| 21           | 1.75 (3H, d, $J=1.04$ Hz)       | 18.3            | 1.65 (3H, s)                 | 19.71           |   |                 |
| 22           | 1.77 (3H, s)                    | 25.8            | 1.69 (3H, s)                 | 19.71           |   |                 |

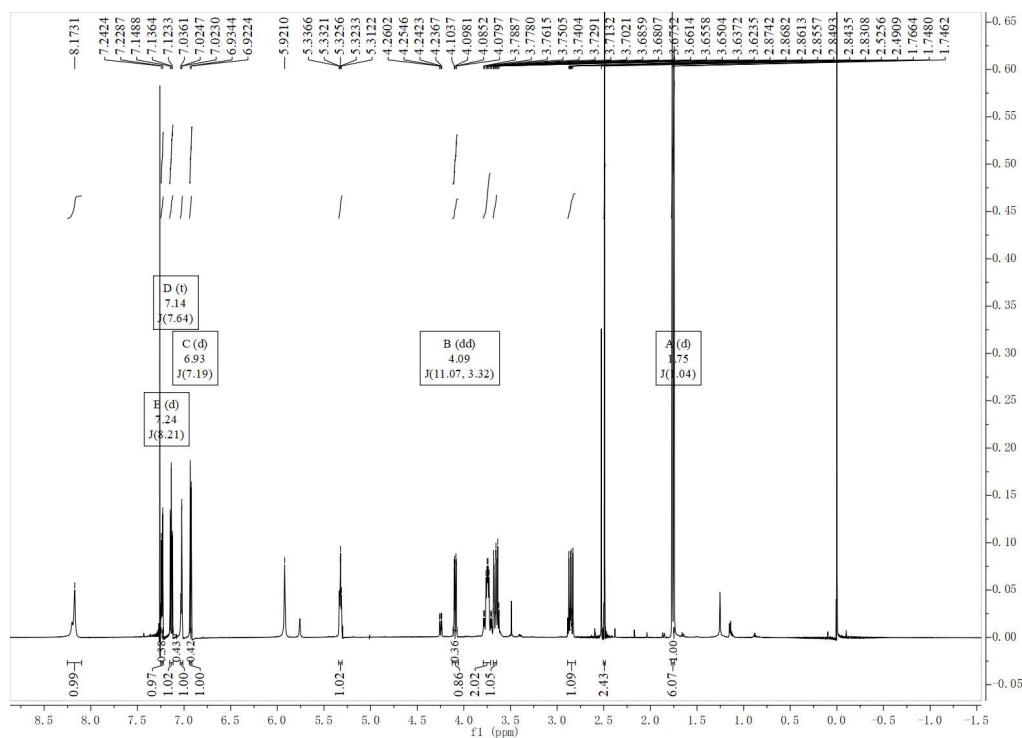


Figure S6.  $^1\text{H}$  NMR spectrum of compound **2** ( $\beta$ -CPA) (600 MHz,  $\text{CDCl}_3$ ).

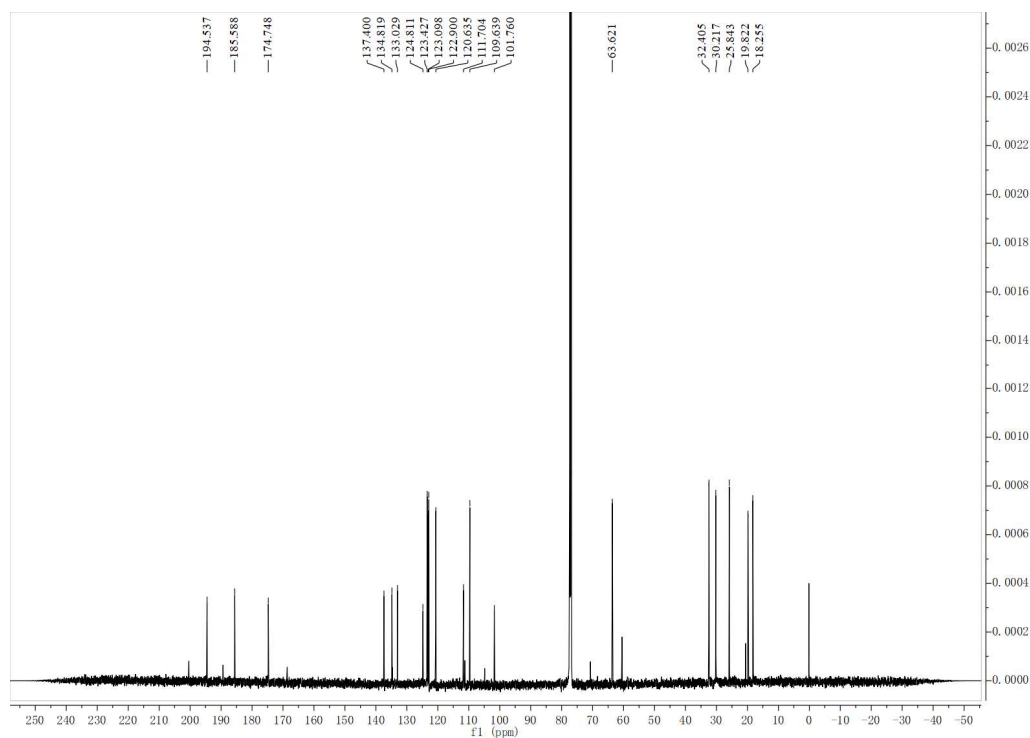


Figure S7.  $^{13}\text{C}$  NMR spectrum of compound **2** ( $\beta$ -CPA) (150 MHz,  $\text{CDCl}_3$ ).

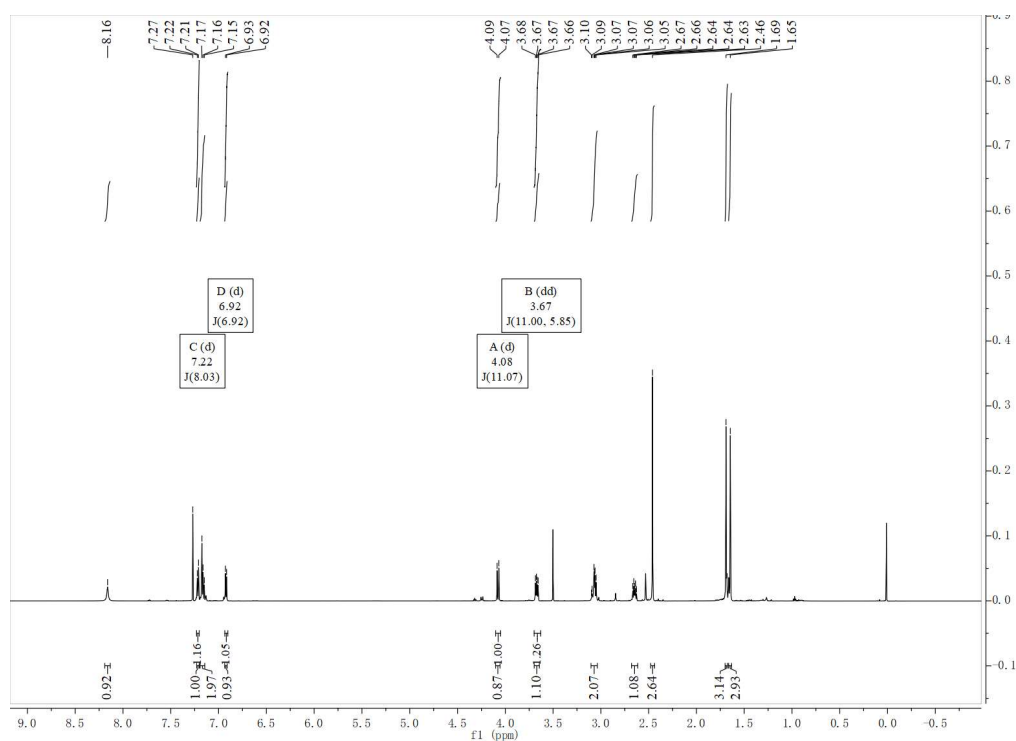


Figure S8.  $^1\text{H}$  NMR spectrum of compound **3** ( $\alpha$ -CPA) (600 MHz,  $\text{CDCl}_3$ ).

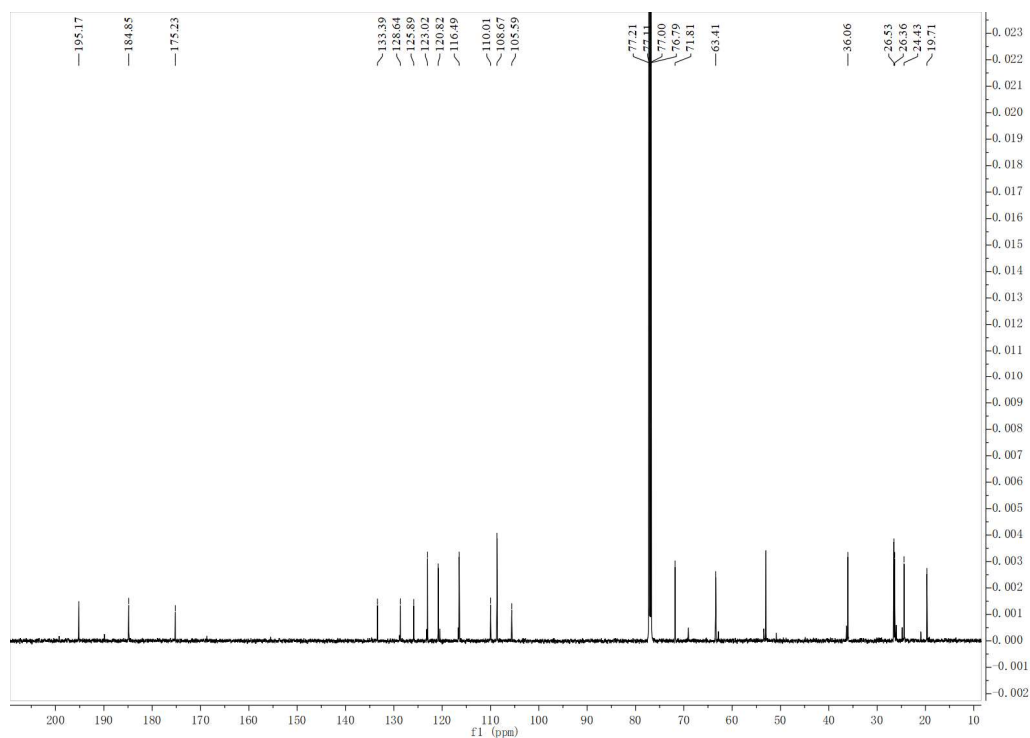


Figure S9.  $^{13}\text{C}$  NMR spectrum of compound **3** ( $\alpha$ -CPA) (150 MHz,  $\text{CDCl}_3$ ).

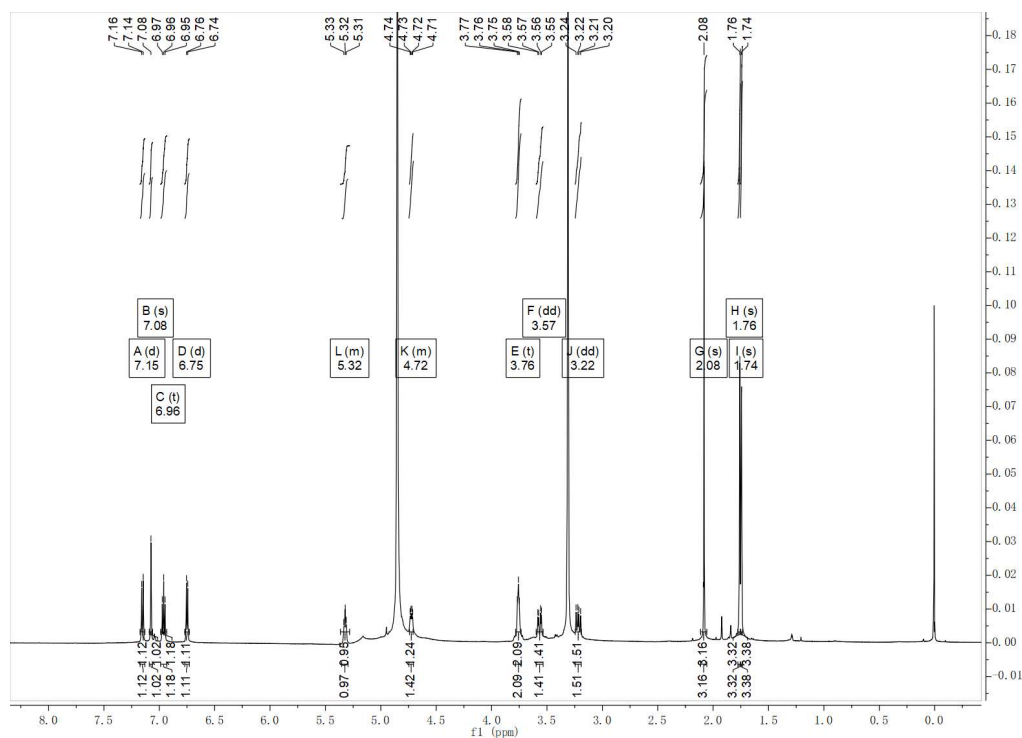


Figure S10. <sup>1</sup>H NMR spectrum of compound 7 (PAATrp) (600 MHz, CDCl<sub>3</sub>).

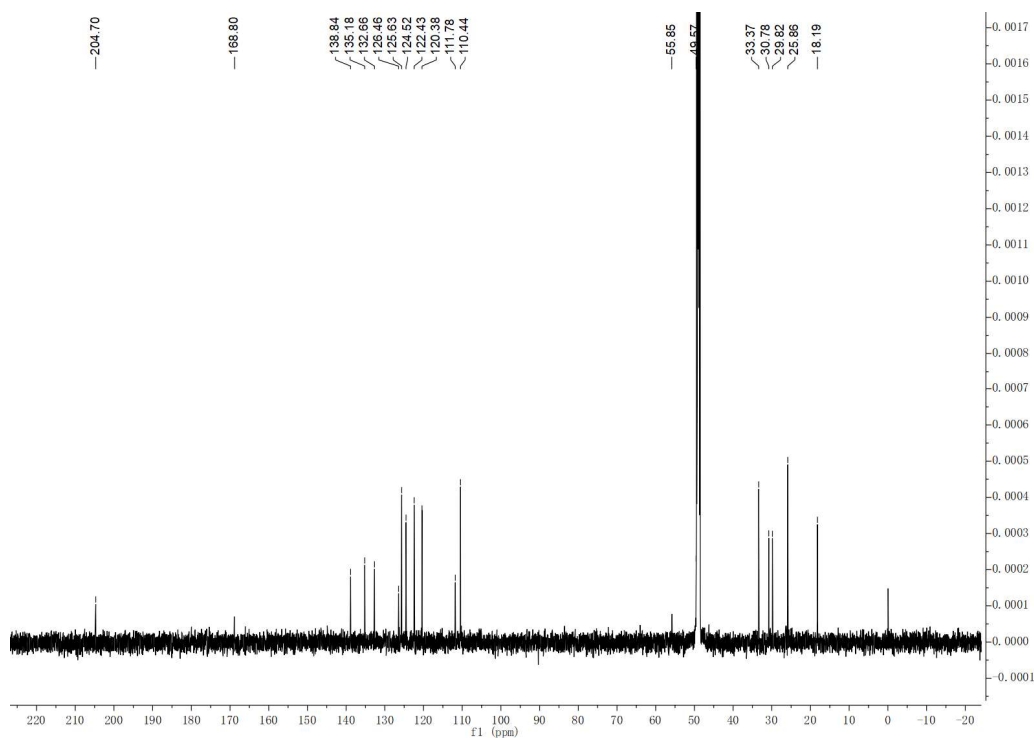


Figure S11. <sup>13</sup>C NMR spectrum of compound 7 (PAATrp) (150 MHz, CDCl<sub>3</sub>).

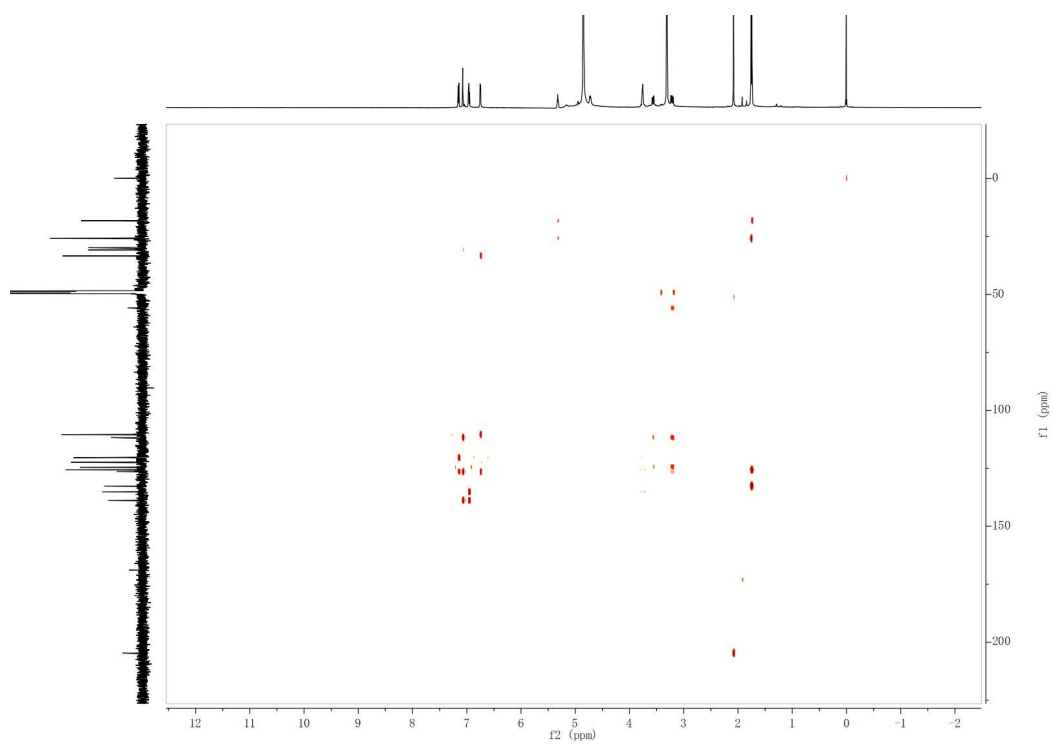


Figure S12. HMBC spectrum of compound **7** (PAATrp) (CD<sub>3</sub>OD).

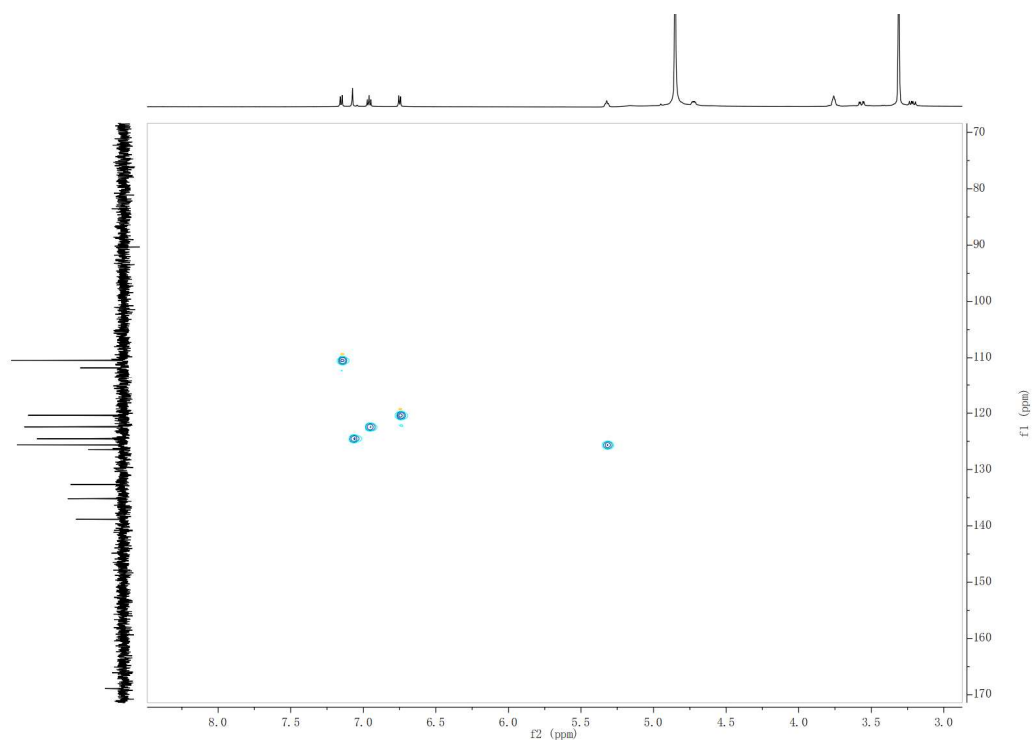


Figure S13. HSQC spectrum of compound **7** (PAATrp) (CD<sub>3</sub>OD).

**>acvA-TE**

CCGATCTTGTTCCTATTGCCGCCAGGGGAGGGGAGGGGCAGAAAGCTACTTTAACAATATCG  
TCAAGCACTTGCCACGACTAATATGGTCGTCTTTAACAATTACTACCTTCACTCCAAGAGT  
CTGAACACGTTTGAAAAGCTAGCTGAGATGTATTTGGGGCACATCCGTCAGATCCAGCCAG  
ACGGGCCTTACCATTTTCATCGGATGGAGTTTGGAGGAACAATCGCGATGGAAATATCGCGA  
CAGCTCGTGGGGCTAGGTTCAACGATTGGTCTTTAGGTATCATTGACACGTATTTCAACGT  
GCCTGGAGCAACGCGGGCAATTGGCCTCGGTGATACTGAGGTCTTGGATCCCATTCATCATA  
TATCCCAACCAGAACCAGCCGATTTCAGTGCCTCCAGCCAGCACAGACTACATCATTTTA  
TTCAAAGCTACTAGGGTGAACGACAAGTTTCAGTCTGAAAACCAGAGGCGTCTGTACGAG  
TACTACGACAAAACATTGCTTAATGATCTCGACTGGTTACTCCCTGGTGCTTCAAACATTTCAT  
CTAGTCCGCCTTGAGGAGGATACTCACTTCTCCTGGGCGACCAATCCACGCCAAATCGCCC  
ACGTTTGTTCACAATCGAGAAATTTCTC

**>fgaPT2**

TCAGTGGAGACCGGAATAATATACCCCTGTCTTTGACAGATCTGGAGGTAAAGCTGTGGGTT  
GACAGGCCGCATCCTGACACTTGACCTTTGATTTCGGATAAGTTTGCAACTGCCCAGTCCCCT  
GTCTCGAAGGATTGAAGATAGACACTCAGATAAGGGGTACGGTCCCTGTAGGAGAAGGATA  
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GTTTCGTAGGTGCGGGCCATTTCACTCCAACCCCGGCGCTCGAAGAACGTCGTCAGGGCAT  
CCGCCACCGCCATGTCGTTTCATGCCGAAGGTTGTGAAATATACTTGCGGCTCTGGGACCGG  
GTCATTCTGGTGCAGGGTGAAATTGGCCATAAGCGGCAGCCTCTCGTCTGGGATAACCCCG  
AGAGGCAGATACGGCGCCGGATAGGACTTCAATCCCGGCGACAGTTGGATCAGATCCCAAA  
GCTCACGCACCAGAGAGAGCCCCCTCTAAAGTGGAAGCGTCTCGGCGCCGTCCGCCAGAG  
TCCACAGGTCTCCATGGCTTCTAGTGAAACCATCTGCTCCAGCAGGTAGATCTTGATTCTC  
GACTTGGCAGGACTGGTCAGATCACAGGACACTAGGCGGGGACTGGCAGTGCTCTTGAA  
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CTGCCAGCCGGCGGACTGAGCCAAAGACCAACTCATGAATTGTCTTGCCGGTGACGACAG  
CTTTGAGAGCCGGGTATATGTACGTCTTAAGTGCAAAGCGGCCATCCTTCAGATCGAGCGCG  
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CTTCTCCAGTGGAAGCAGGTGCTGCAGGCTCTCCAGATGGCGTGCGTATTGAATGGGTCT  
TTATCTGTTCCGGTATGTTGATTGATCGGCTCGAATGTGTATCTCACTATTGAATTAGAGCAAT  
TTAGGCTCAATTGCAACGGAGTGCCGTATCGAGTGAGGATGCTCAACCAGCGGGGGCGCT  
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ACTGACAAGGTGTGGTGTAGTTGGCAGTTTCCAGCATTTTGTCAAACATCGGGGCAGTGCT  
GTGCCACCACAGCTTCTGATCTTCATTATCGAATCTAAAGGCGCGACTAAGAACTCGATAGG  
CCTCCGCACTGGAGGCATTGGCTGCCTTCAT