# StnK2 Catalysing A Pictet-Spengler Reaction Involved in the Biosynthesis of Antitumor Reagent Streptonigrin†

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## Content

## Experimental Details

### Cloning, expression, and purification of StnK2 and TK

The genes encoding StnK2 and TK were amplified by PCR using the following primers: *stnK2_F*: CCTACATATGCGCCAAATCGAGATCG (NdeI site underlined); *stnK2_R*: CCACTCGAGTCAGGCATGCGCACTC (XhoI site underlined) for *stnK2* and *TK_F*: CCTTACATATGATGTCCTCACGTAAAGAGC (NdeI site underlined); *TK_R*: CCTTACTCGAGTTACAGCAGTTCTTTTGCTTTCG (XhoI site underlined). PCR amplifications were performed on a Veriti thermal cycler (Applied Biosystems, Carslbad, CA) using Taq DNA polymerase or KOD-plus high-fidelity polymerase, which were purchased from Takara Co. Ltd Company (Dalian, China). Restriction enzymes (NdeI and XhoI) and DNA ligase were purchased from Fermentas (Thermo Fisher Scientific Inc) or NEB companies (Gene, England) to treat PCR products. The resulting fragments were cloned into pET28a digested with the same restriction enzymes to yield the expression plasmid pLS2154-*stnK2* and pLS2154-*TK*. The *E. coli* BL21(DE3)pLysS cells harboring the expression plasmid were cultured at 37 °C and 220 rpm in LB medium supplemented with 50 μg $mL^{-1}$ kanamycin to $OD_{600}$ between 0.4 and 0.6. The culture was then incubated on ice for 10 min before addition of 0.4 mM (final concentration) IPTG to induce protein expression. The cells were further cultured at 16 °C for 18-20 h, and then were harvested by centrifugation (3,500 rpm, 15 min, 4°C). The harvested cells were re-suspended in 40 mL buffer A (50 mM Tris-HCl, pH 8.0, 0.5 M NaCl, and 10% glycerol) and lysed by sonication on ice for 40 min. Cellular debris was removed by centrifugation (12,500 rpm, 45 min, 4°C), and the supernatant was used to purify the protein by nickel-affinity chromatography using standard protocols. The protein was eluted with increasing gradient of buffer B (500 mM imidazole in buffer A). Purified proteins were concentrated and exchanged into buffer C (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, and 5% glycerol) with the centriprep filters (Amicon). The protein purity was >90 % judged by SDS-PAGE analysis and was stored in buffer C at -80 °C. Protein concentration was determined by Bradford assay using bovine serum albumin as a standard.

**Heterologous production and isolation of 4-fluoro-(2*S*,3*S*)-β-methyltryptophan, 5-fluoro-(2*S*,3*S*)-β-methyltryptophan or 6-fluoro-(2*S*,3*S*)-β-methyltryptophan.**

The large amounts of 4-fluoro-(2*S*,3*S*)-β-methyltryptophan, 5-fluoro-(2*S*,3*S*)-β-methyltryptophan or 6-fluoro-(2*S*,3*S*)-β-methyltryptophan were prepared by biotransformation of 4-fluoro-L-tryptophan, 5-fluoro-L-tryptophan or 6-fluoro-L-tryptophan according to the reported method, respectively.[1] The column used for purification was flash column packed with reverse phase silica gel (YMG*GEL, ODS-A-HG, 50 μm, 2 × 15 cm). The purity was checked by HPLC analysis.

For structural elucidation, $^{1}H$ and $^{13}C$ NMR spectra were recorded on Bruker AV instruments.

**In vitro biochemical assays and product analysis.**

The reaction mixture (100 μl) for StnK2 contained 10 μM StnK2, 15 μM TK, 1.5 mM fructose-6-phosphate and 1.5 mM glyceraldehyde 3-phosphate, 1 mM substrate such as (2*S*,3*S*)-β-methyl tryptophan, (2*S*,3*R*)-β-methyl tryptophan, L-tryptophan, D-tryptophan, 4-fluoro-L-tryptophan, 5-fluoro-L-tryptophan, 6-fluoro-L-tryptophan, 4-fluoro-(2*S*,3*S*)-β-methyl tryptophan, 5-fluoro-(2*S*,3*S*)-β-methyl tryptophan, 6-fluoro-(2*S*,3*S*)-β-methy ltryptophan, 5-hydroxyl-L-tryptophan, and 5-hydroxyl-tryptamine in Citric acid /disodium hydrogen phosphate buffer (pH 8.0) and was incubated at 30 °C for 1 h. Reactions were quenched by being frozen in -80 °C quickly and then lyophilized. 100 μl methanol was used to re-dissolve the reaction mixture and 80 μl supernatant after centrifugation (12000 rpm, 10 min) for removal of the proteins was subjected to HPLC analysis.

The column used for HPLC and HR-LC-Q-TOF detection was a C18 column (5 μm, 4.6 × 150 mm). The flow rate was 0.3 ml/min for HPLC. The mobile phase was comprised of acetonitrile and water containing 0.1% (v/v) formic acid ((v/v): 5:95 to 40:60, 0 to 15 min; 40:60 to 100:0, 15 to 20 min; 100:0, 20 to 25 min; 100:0 to 5:95, 25 to 26 min; 5:95, 26 to 30 min) with detection at 254 nm.

**Preparative scale in vitro reaction for structural elucidation of 5.**

A solution with a total volume of 100 ml composed of 10 μM StnK2, 15 μM TK, 1.5 mM fructose-6-phosphate and 1.5 mM glyceraldehyde 3-phosphate, 1 mM (2*S*,3*S*)-β-methyltryptophan in Citric acid /disodium hydrogen phosphate buffer (pH 8.0) and was incubated at 30°C for 6 h. The reaction was monitored by HPLC analysis. After 6 h incubation, the reaction was quenched by being frozen at -80 °C and then lyophilized to dryness. 1 ml 10% (v/v) methanol was used to re-dissolve the reaction mixture and the supernatant after centrifugation (15000 rpm, 10 min) was used to purify the products. The column used for purification was flash column packed with reverse phase silica gel (YMG*GEL, ODS-A-HG, 50 μm, 2 × 15 cm). The purity was checked by HPLC analysis.

For structural elucidation, $^{1}H$, $^{13}C$, $^{1}H$-$^{1}H$ COSY, HMBC, HSQC and NOESY spectra were recorded on Bruker AV instruments.

**Kinetic analyses for StnK2 with (2*S*, 3*S*)-β-methyl tryptophan, 5-fluoro-L-tryptophan, 6-fluoro-L-tryptophan and 5-fluoro-(2*S*, 3*S*)-β-methyl tryptophan and 6-fluoro-(2*S*,3*S*)-β-methyl tryptophan**

To obtain the kinetic parameters of StnK2 for the substrate (2*S*,3*S*)-β-methyl tryptophan, 5-fluoro-L-tryptophan, 6-fluoro-L-tryptophan and 5-fluoro-(2*S*,3*S*)-β-methyl tryptophan and 6-fluoro-(2*S*,3*S*)-β-methyl tryptophan, reactions were performed at 30°C in Citric acid /disodium hydrogen phosphate buffer (pH 8.0) for 1 h for (2*S*,3*S*)-β-methyl tryptophan, 5-fluoro-L-tryptophan, 6-fluoro-L-tryptophan and 3 h for 5-fluoro-(2*S*,3*S*)-β-methyl tryptophan, 6-fluoro-(2*S*,3*S*)-β-methyl tryptophan. The yields of the products can be measured by using HPLC. When (2*S*,3*S*)-β-methyltryptophan was used as the substrate, the reaction mixture contained 5 μM StnK2, 10 μM TK, 1 mM fructose-6-phosphate and 1 mM glyceraldehyde 3-phosphate and varying concentrations of (2*S*,3*S*)-β-methyltryptophan (0 μM, 50 μM, 100 μM, 200 μM, 350 μM, 500 μM, 1000 μM, 2000 μM). When 5-fluoro-L-tryptophan or 6-fluoro-L-tryptophan was used as the substrate, the reaction mixture contained 5 μM StnK2, 10 μM TK, 1mM fructose-6-phosphate and 1mM glyceraldehyde-3phosphate and varying concentrations of 5-fluoro-L-tryptophan, 6-fluoro-L-tryptophan and 5-fluoro-(2*S*,3*S*)-β-

methyl tryptophan and 6-fluoro-(2*S*,3*S*)-β-methyl tryptophan (0 μM, 20 μM, 40 μM, 80 μM, 100 μM, 500 μM, , 1000 μM, 2000 μM). Each assay was carried out in triplicate. The $K_m$ and $k_{cat}$ values were calculated using GraphPad Prism 6.

References

[1] Y. Zou, Q. Fang, H. Yin, Z. Liang, D. Kong, L. Bai, Z. Deng and S. Lin, Angewandte Chemie, 2013, 52, 12951-12955.

## Tables.

**Table S1.** NMR data of **5** ($D_2O$).

| No. | $\delta_H$ (multi, *J* in Hz) | $\delta_C$ | HMBC | $^1H,^1H$-COSY |
|---|---|---|---|---|
| 2 | | 129.0 (s) | | |
| 3 | | 115.6 (s) | | |
| 4 | | 127.6 (s) | | |
| 5 | 7.75 (d, 12.1) | 122.4 (d) | C-3, C-7, C-9 | H-6 |
| 6 | 7.15 (t, 11.4) | 122.5 (d) | C-4, C-8 | H-5, H-7 |
| 7 | 7.27 (t, 10.1) | 125.3 (d) | C-5, C-9 | H-6, H-8 |
| 8 | 7.50(d, 12.2) | 114.8 (d) | C-4, C-6 | H-7 |
| 9 | | 139.6 (s) | | |
| 10 | 3.48 (m) | 32.8 (d) | C-4 | H-17, H-11 |
| 11 | 3.60 (d, 15.7) | 67.0 (d) | C-13, C-17 | H-10 |
| 13 | 5.10(brs) | 57.3(d) | C-3,C-11, | H-14 |
| 14 | 4.43 (m) | 70.4 (d) | C-11 | H-15, H-13 |
| 15 | 3.90 (m) | 73.8(d) | C-14 | H-16, H-14 |
| 16 | 4.09(m) | 68.5 (t) | C-14 | H-15 |
| 17 | 1.59 (d, 9.7) | 20.1 (q) | C-3, C-11 | H-10 |
| 18 | | 176.2 (s) | | |

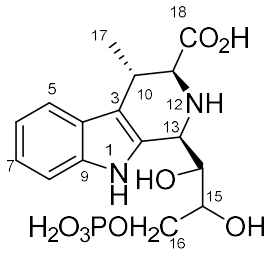


**Table S2.** NMR data of 4-fluoro-(2*S*,3*S*)-β-methyl tryptophan ($CD_3OD$).

| No. | $\delta_H$ (multi, *J* in Hz) | $\delta_C$ |
|---|---|---|
| 1′ | | 174.3 |
| 2′ | 3.83(1H,d,8.5) | 61.9 |
| 3′ | 3.48(1H,dd,7.1,7.2) | 36.2 |
| 4′ | 1.50(3H,d,6.8) | 19.6 |
| 1 | | |
| 2 | 7.20 | 125.1 |
| 3 | | 115.0 |
| 3a | | 115.8 |
| 4 | | 157.5 |
| 5 | 6.72(1H,m) | 105.2 |
| 6 | 7.05(1H,m) | 123.3 |
| 7 | 7.20(1H,m) | 111.2 |
| 7a | | 141.8 |

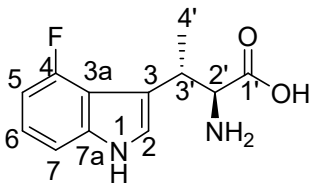


**Table S3.** NMR data of 5-fluoro-(2*S*,3*S*)-β-methyl tryptophan ($CD_3OD$).

| No. | $\delta_H$ (multi, *J* in Hz) | $\delta_C$ |
|---|---|---|
| 1′ | | 173.9 |
| 2′ | 3.78(1H,d,6.6) | 61.2 |
| 3′ | 3.58(1H,dd,7.0,7.0) | 34.8 |
| 4′ | 1.53(3H,d,7.2) | 19.8 |
| 1 | | |
| 2 | 7.25(1H,m) | 125.9 |
| 3 | | 115.3 |
| 3a | | 128.0 |
| 4 | 7.40(1H,m) | 113.4 |
| 5 | | 159.1 |
| 6 | 6.87(1H,m) | 111.1 |
| 7 | 7.48(1H,m) | 104.7 |
| 7a | | 135.2 |

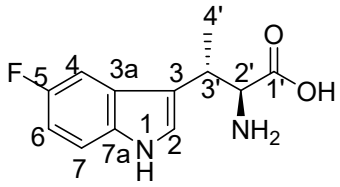


**Table S4.** NMR data of 6-fluoro-(2*S*,3*S*)-β-methyl tryptophan ($D_2O$).

| No. | $\delta_H$ (multi, *J* in Hz) | $\delta_C$ |
|---|---|---|
| 1′ | | 182.4 |
| 2′ | 3.18(1H,d,6.8) | 61.7 |
| 3′ | 3.11(1H,dd,7.0,7.0) | 35.8 |
| 4′ | 1.15(3H,d,7.0) | 18.5 |
| 1 | | |
| 2 | 7.0(1H,m) | 123.3 |
| 3 | | 116.2 |
| 3a | | 123.3 |
| 4 | 7.43(1H,m) | 119.9 |
| 5 | 6.71(1H,m) | 107.2 |
| 6 | | 159.5 |
| 7 | 7.0(1H,m) | 97.5 |
| 7a | | 136.1 |

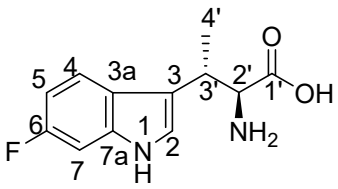
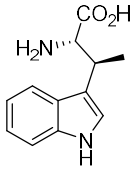
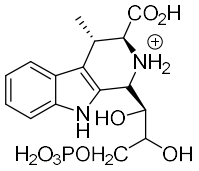
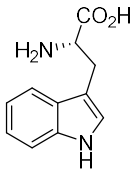
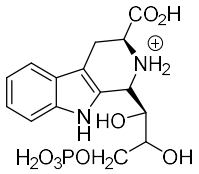
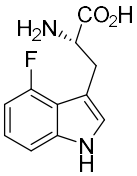
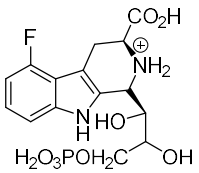
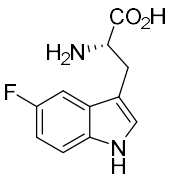
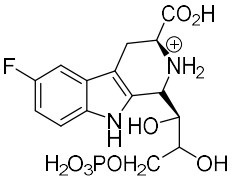
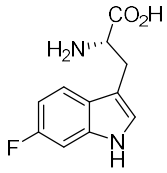
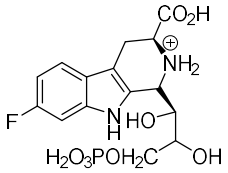
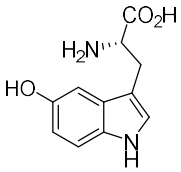
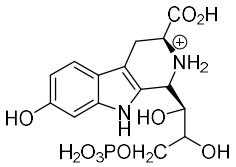
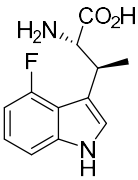
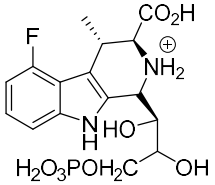
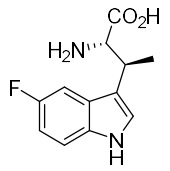
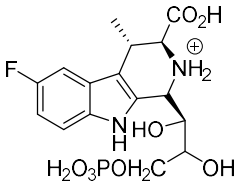
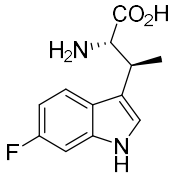
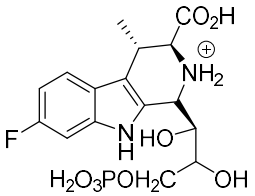


**Table S5.** HR-MS data for the products in the biochemical assays.

| Substrate | *m/z* [M+H]$^+$ | | Chemical formula | product |
|---|---|---|---|---|
| | Calcd | Detected | | |
|  | 401.1108 | 401.1105 | $C_{16}H_{22}N_2O_8P^+$ |  |
|  | 387.0951 | 387.0923 | $C_{15}H_{20}N_2O_8P^+$ |  |
|  | 405.0858 | 405.0814 | $C_{15}H_{20}FN_2O_8P^+$ |  |
|  | 405.0858 | 405.0812 | $C_{15}H_{20}FN_2O_8P^+$ |  |
|  | 405.0858 | 405.0811 | $C_{15}H_{20}FN_2O_8P^+$ |  |
|  | 403.0901 | 403.0881 | $C_{15}H_{20}N_2O_9P^+$ |  |
|  | 419.1014 | 419.0900 | $C_{16}H_{21}FN_2O_8P^+$ |  |

| | | | | |
|---|---|---|---|---|
|  | 419.1014 | 419.0979 | $C_{16}H_{21}FN_2O_8P^+$ |  |
|  | 419.1014 | 419.0990 | $C_{16}H_{21}FN_2O_8P^+$ |  |

## Figures

**Figure S1.** Amino acids alignment of StnK2 and other homologous proteins. StnK2 from *S. flocculus* CGMCC 4.1223; McbB from *Marinactinospora thermotolerans* SCSIO 00652; Mikimopine synthase from *Nicotiana glauca*; Cucumopine synthase from *Beauveria bassiana* ARSEF 2860.

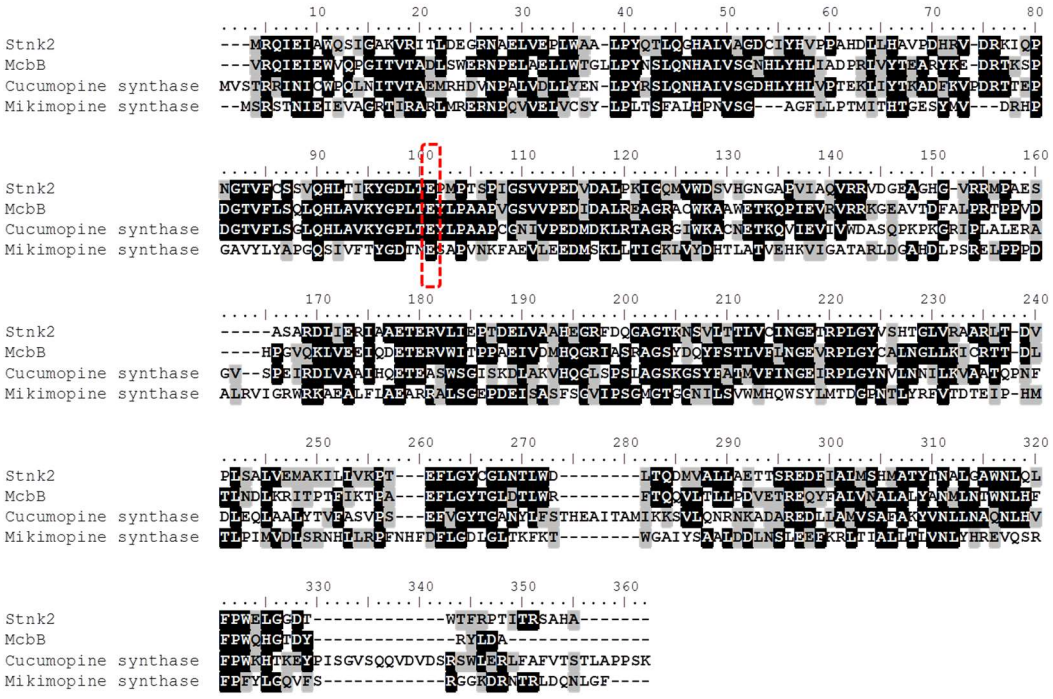



**Figure S2.** SDS-PAGE of StnK2 and TK.

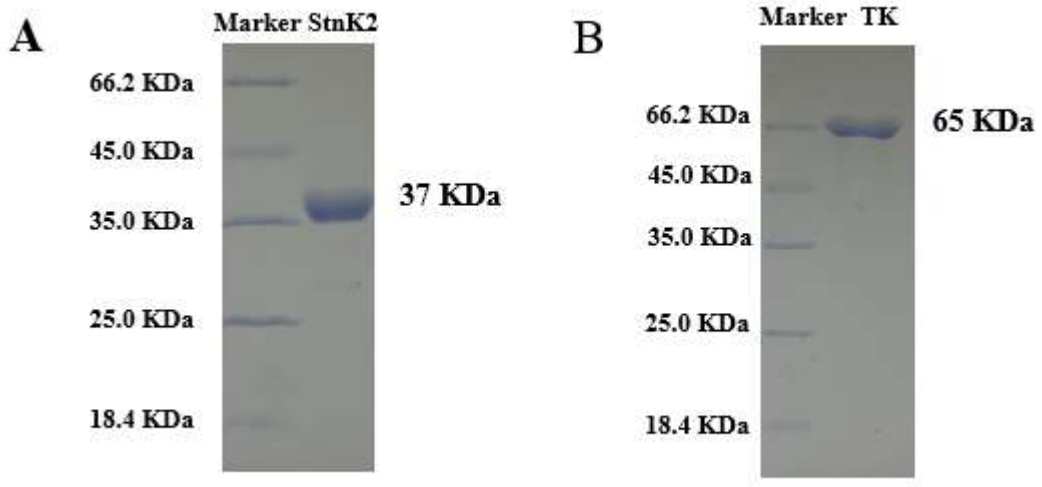


**Figure S3.** Substrate specificity of StnK2 for aldehydes. HPLC profiles (detection at 254 nm) of the reactions: (i) fructose-6-phosphate, glyceraldehyde 3-phosphate and TK incubated with (2*S*,3*S*)-β-methyl tryptophan and StnK2; (ii) Methylglyoxal incubated with (2*S*,3*S*)-β-methyl tryptophan and StnK2; (iii) Ethyl glyoxalate incubated with (2*S*,3*S*)-β-methyl tryptophan and StnK2; (iv) Erythrose incubated with (2*S*,3*S*)-β-methyl tryptophan and StnK2; (v) (2*S*,3*S*)-β-methyl tryptophan standard. *indicates the PS product.

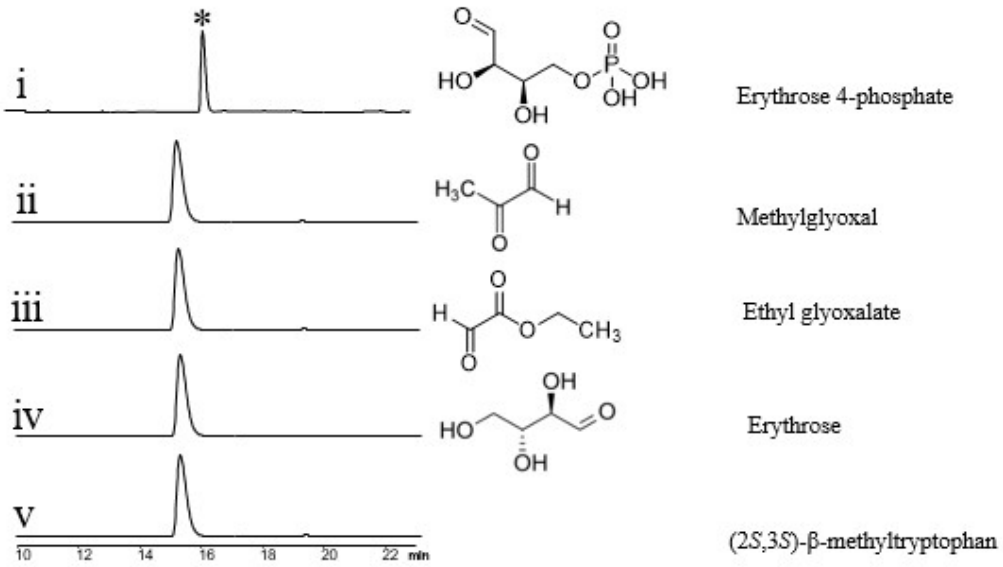


**Figure S4.** HR-MS data of **5**.

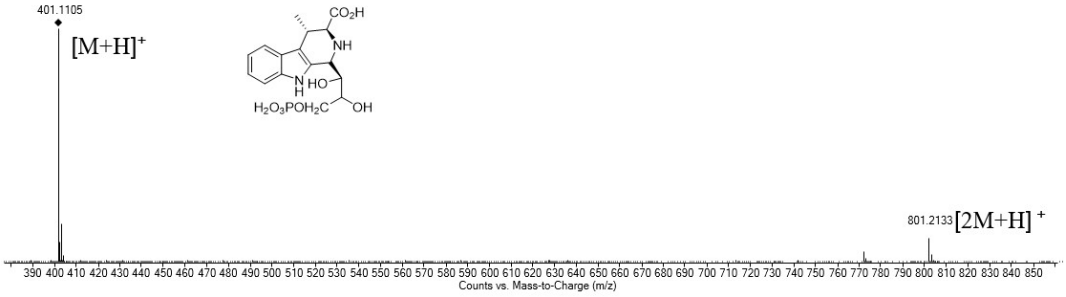



**Figure S5.** Screening the optimal buffer for StnK2/TK reaction.

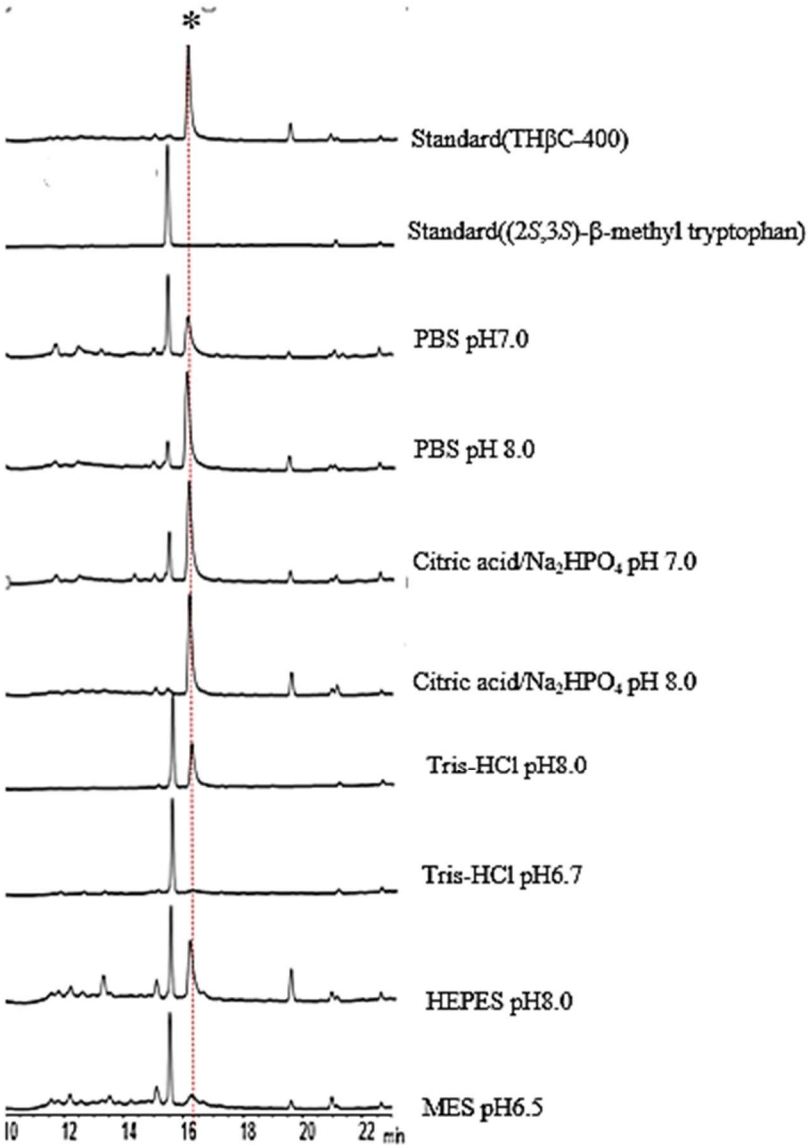


**Figure S6.** 1D NMR spectra of **5**. A. $^{1}$H NMR spectrum (500 MHz, $D_2O$). B. $^{13}$C NMR spectrum (125 MHz).

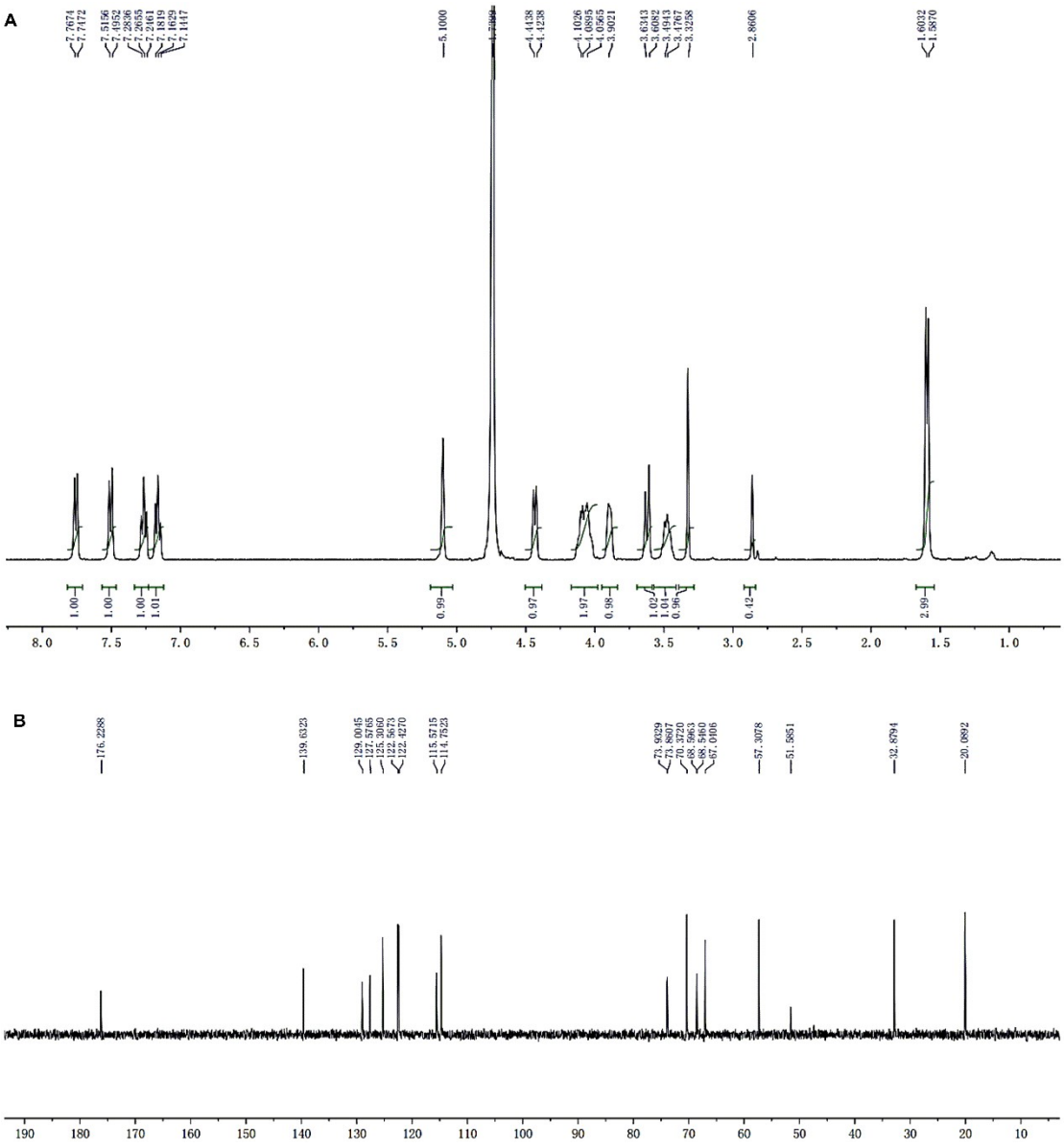
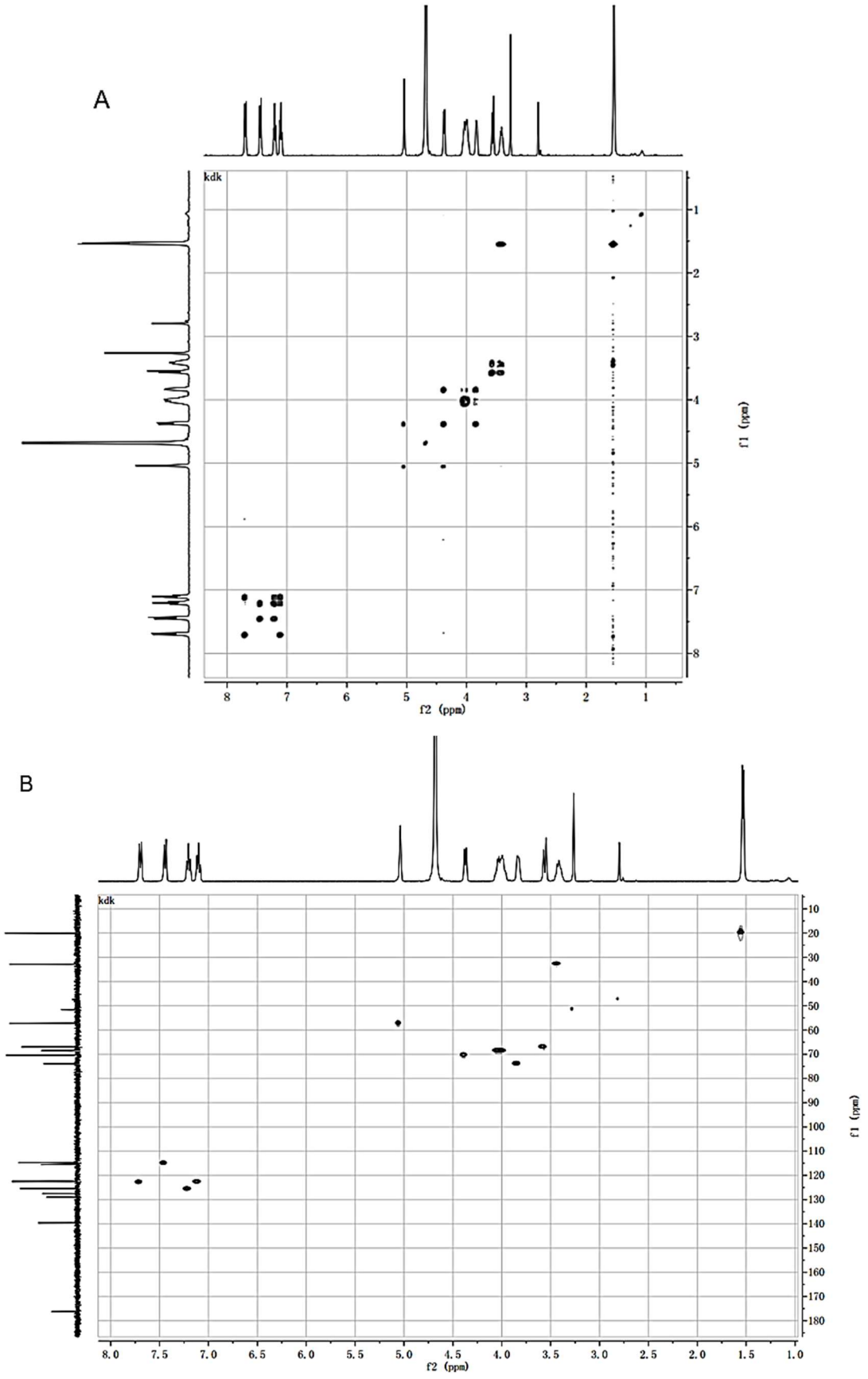
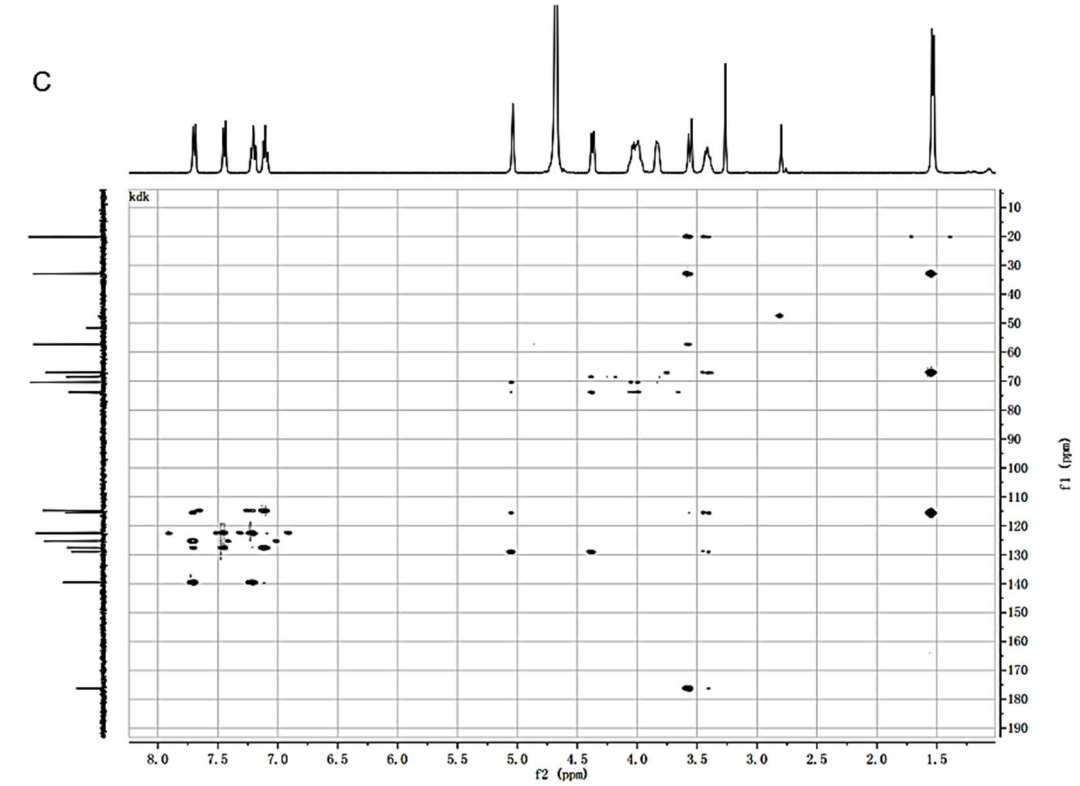


**Figure S7.** 2D NMR spectra of **5**. A, $^{1}H$, $^{1}H$-COSY spectrum; B, HSQC spectrum; C, HMBC spectrum; D. NOESY spectrum.




C
kdk
f2 (ppm)
f1 (ppm)

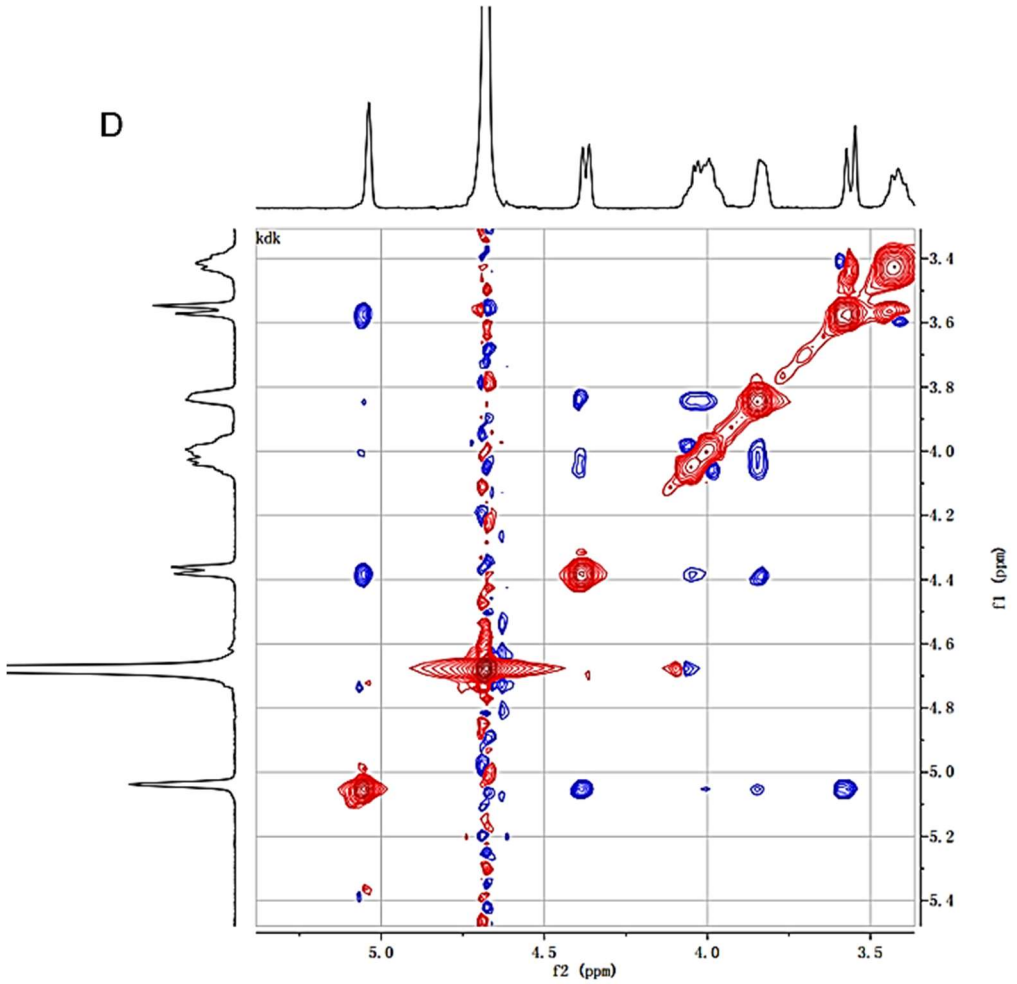

D
kdk
f2 (ppm)
f1 (ppm)

**Figure S8.** HR-MS data of 4-fluoro-(2*S*,3*S*)-β-methyl tryptophan (A), 5-fluoro-(2*S*,3*S*)-β-methyl tryptophan (B) and 6-fluoro-(2*S*,3*S*)-β-methyl tryptophan (C).

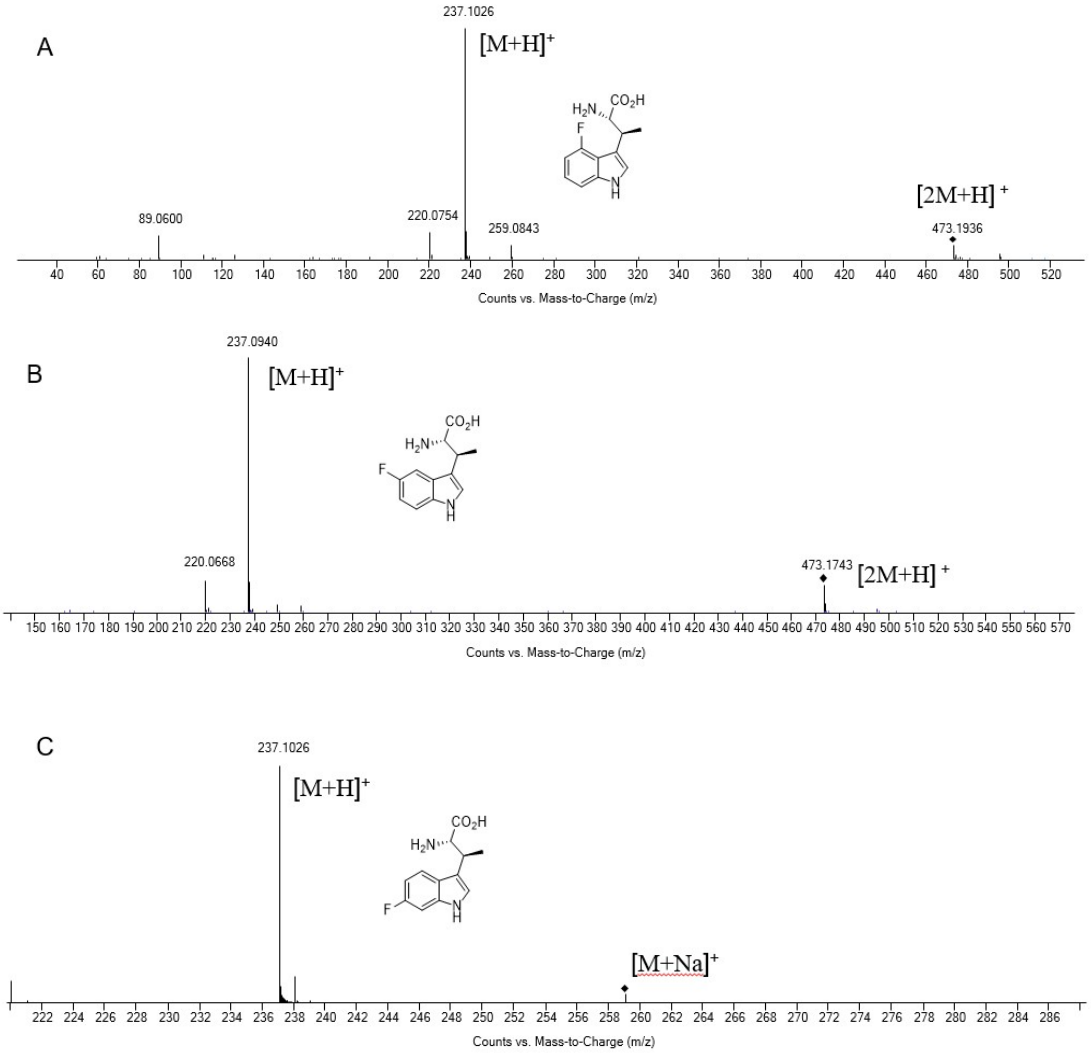


**Figure S9.** 1D NMR spectra of 4-fluoro-(2*S*,3*S*)-β-methyl tryptophan . A, $^{1}H$ NMR spectrum; B, $^{13}C$ NMR spectrum.

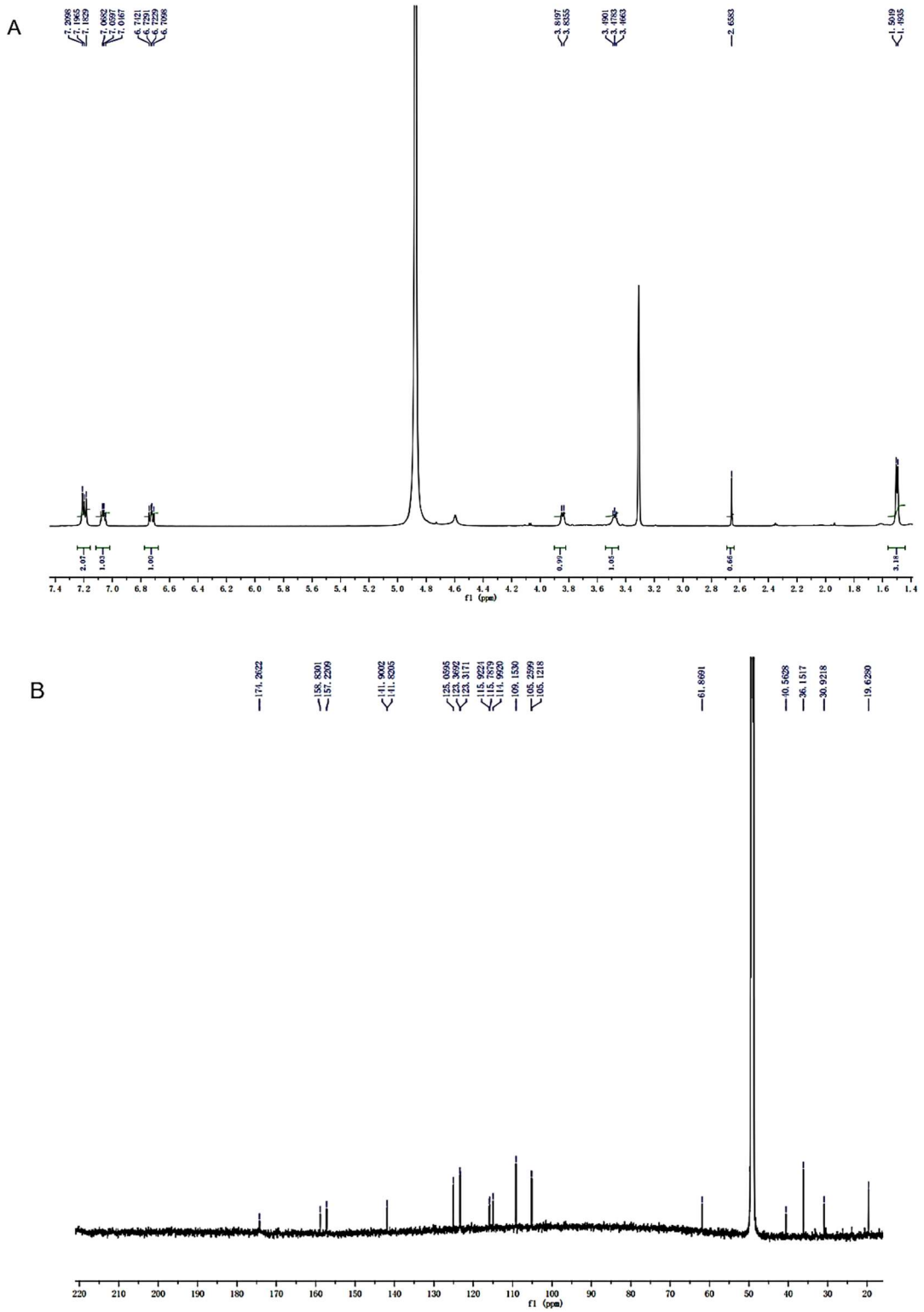


**Figure S10.** 1D NMR spectra of 5-fluoro-(2*S*,3*S*)-β-methyl tryptophan. A, $^{1}H$ NMR spectrum; B, $^{13}C$ NMR spectrum.

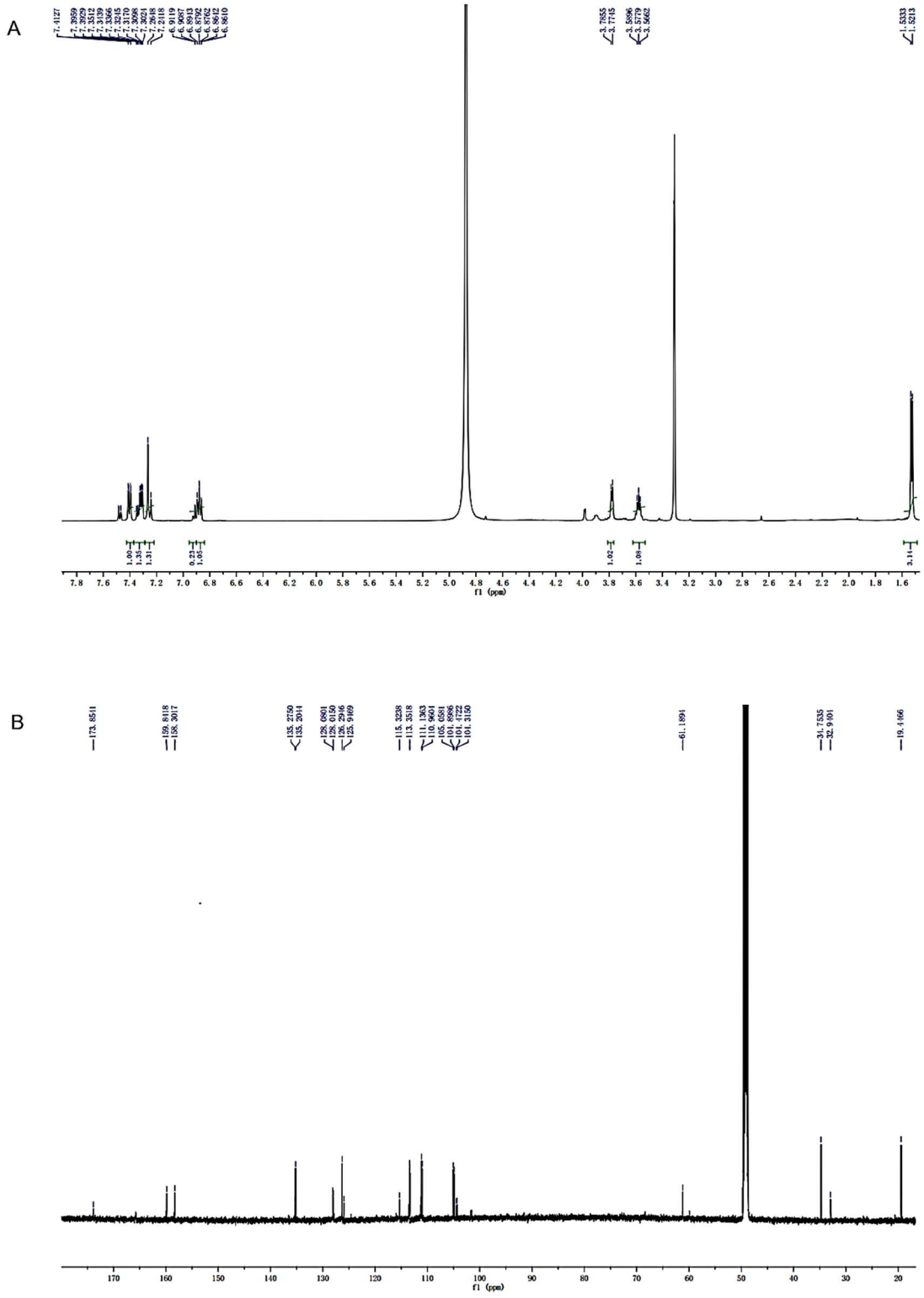


**Figure S11.** 1D NMR spectra of 6-fluoro-(2*S*,3*S*)-β-methyl tryptophan. A, $^{1}H$ NMR spectrum; B, $^{13}C$ NMR spectrum.

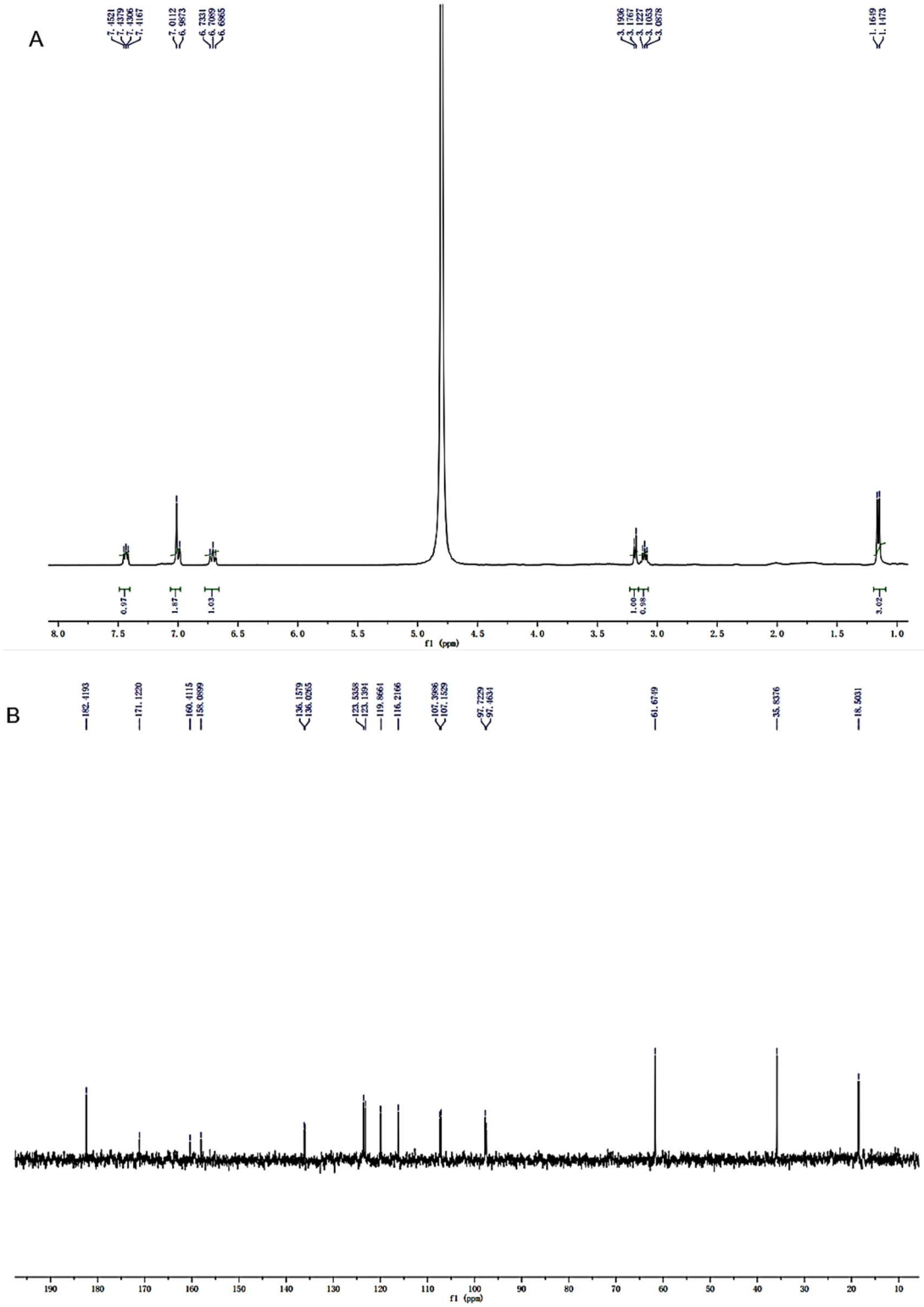


**Figure S12.** HR-MS data of the products of 4-fluoro-(2*S*,3*S*)-β-methyl tryptophan (A), 5-fluoro-(2*S*,3*S*)-β-methyl tryptophan (B), 6-fluoro-(2*S*,3*S*)-β-methyl tryptophan(C).

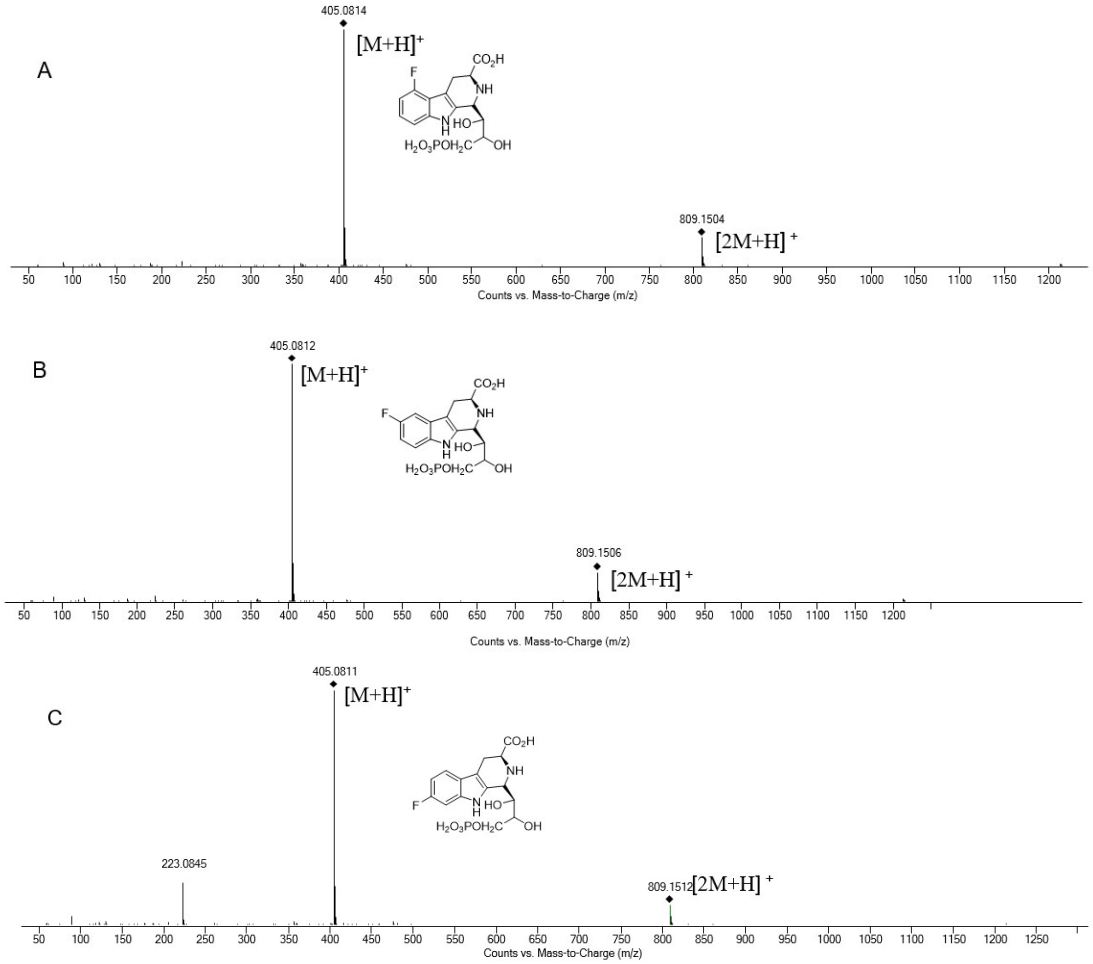



**Figure S13.** Kinetic analyses for StnK2.

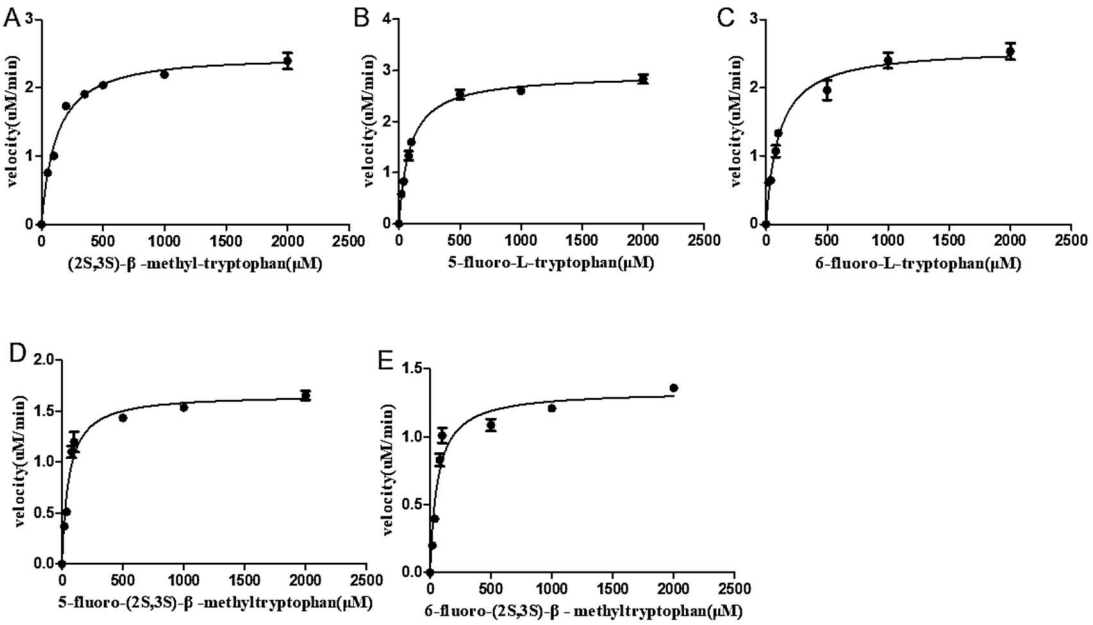
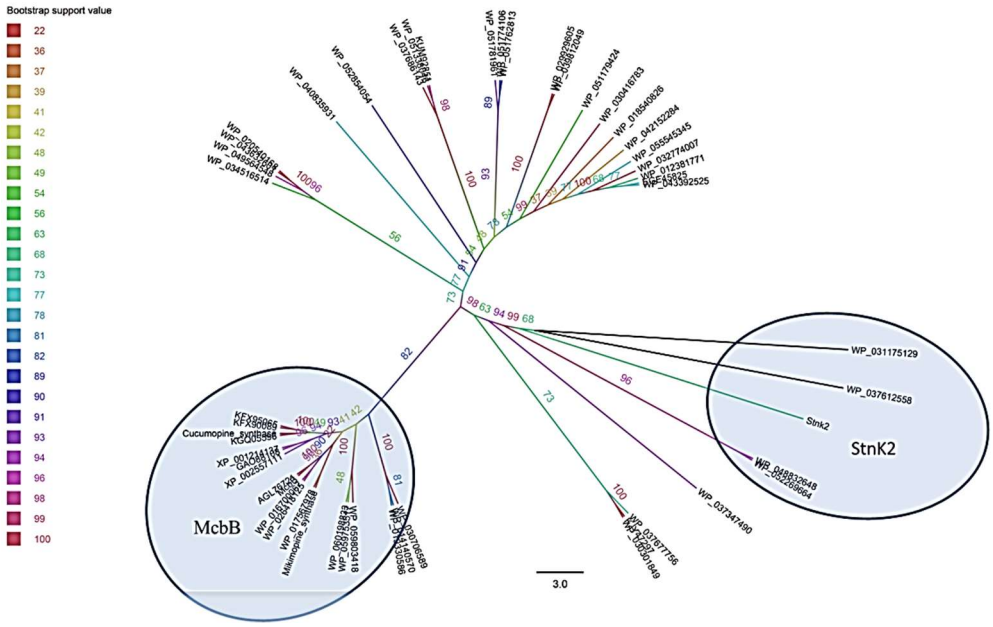


**Figure S14.** A 1000 bootstrap RAxML phylogenetic tree analysis of StnK2 and other homologues.




## References

1. Y. Zou, Q. Fang, H. Yin, Z. Liang, D. Kong, L. Bai, Z. Deng and S. Lin, Angewandte Chemie, 2013, 52, 12951-12955.