Supporting information and experimental section for:

A super versatile flexizyme system with phenol esters for genetic code reprogramming

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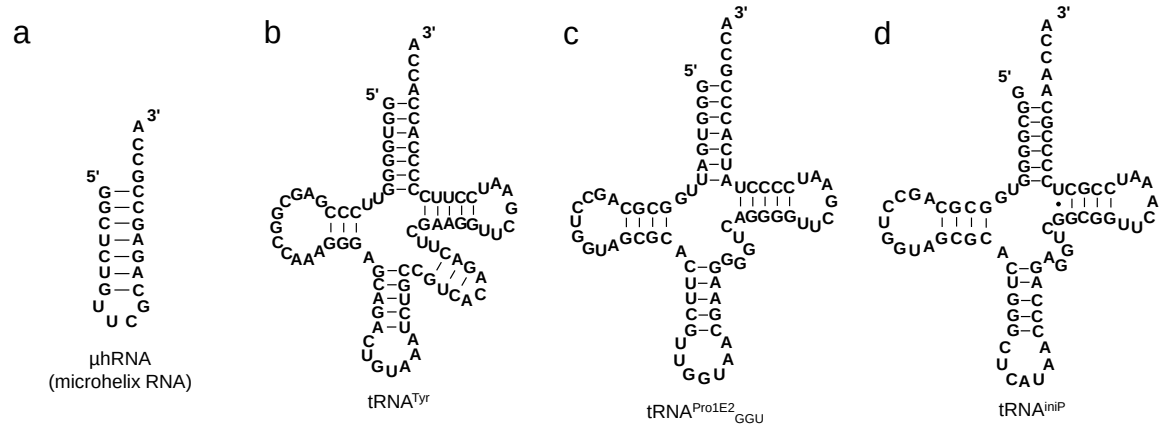
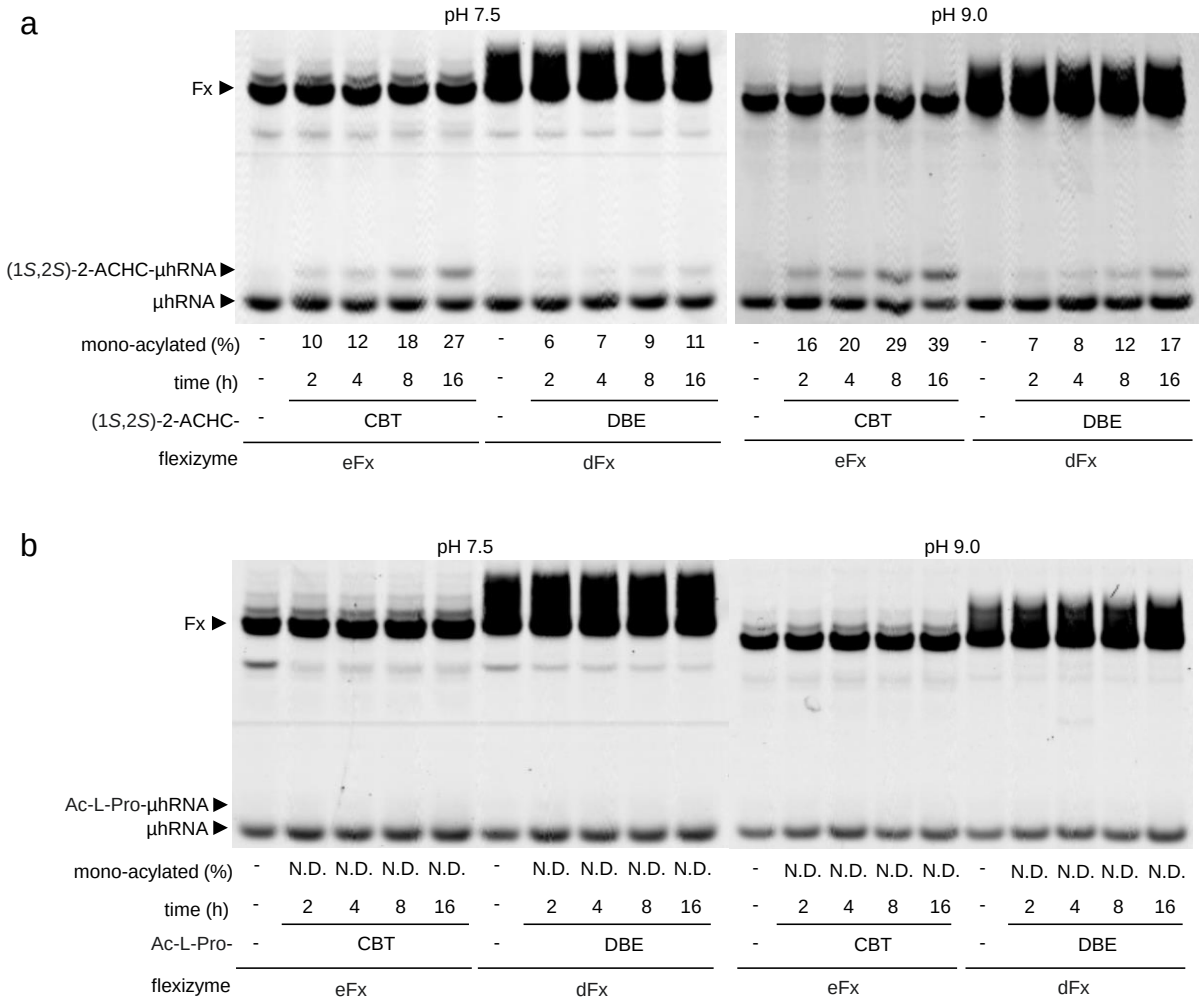
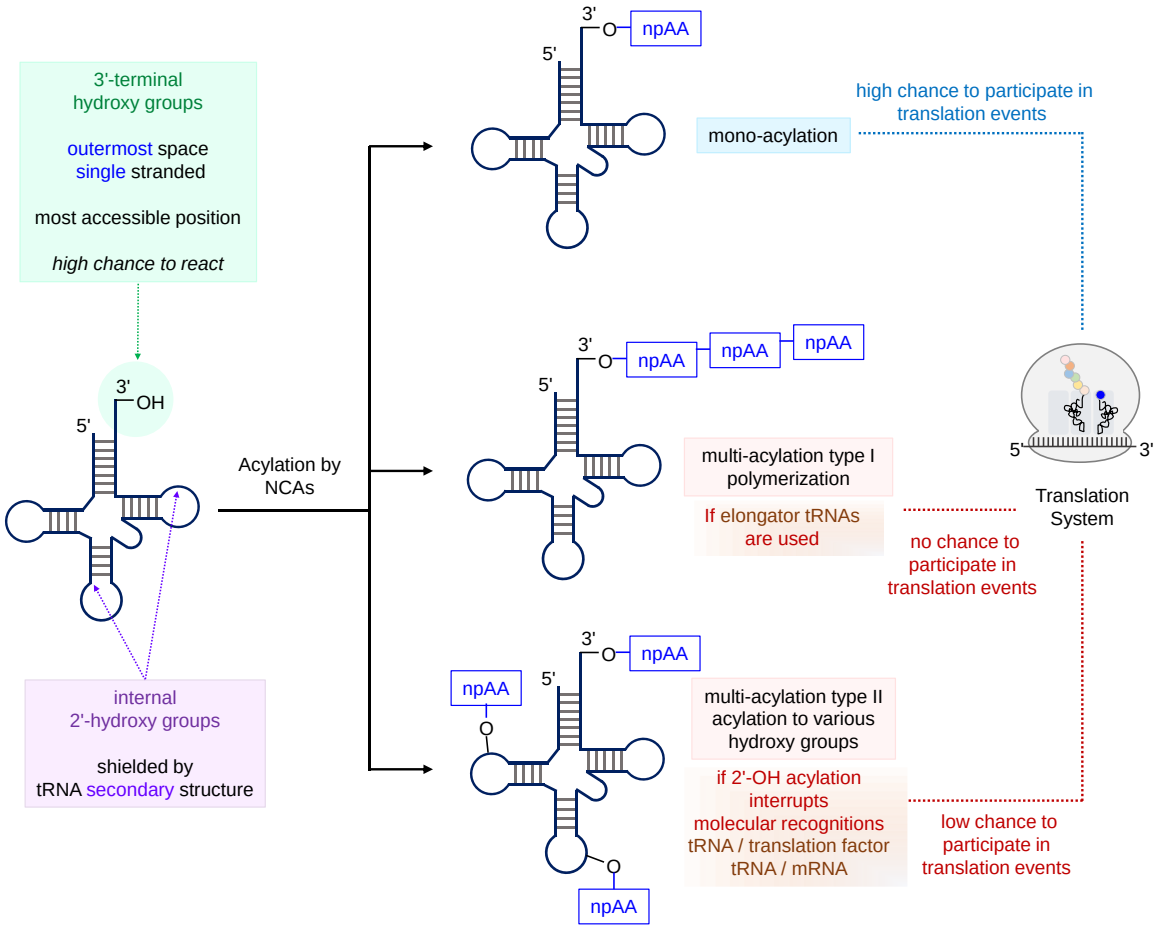


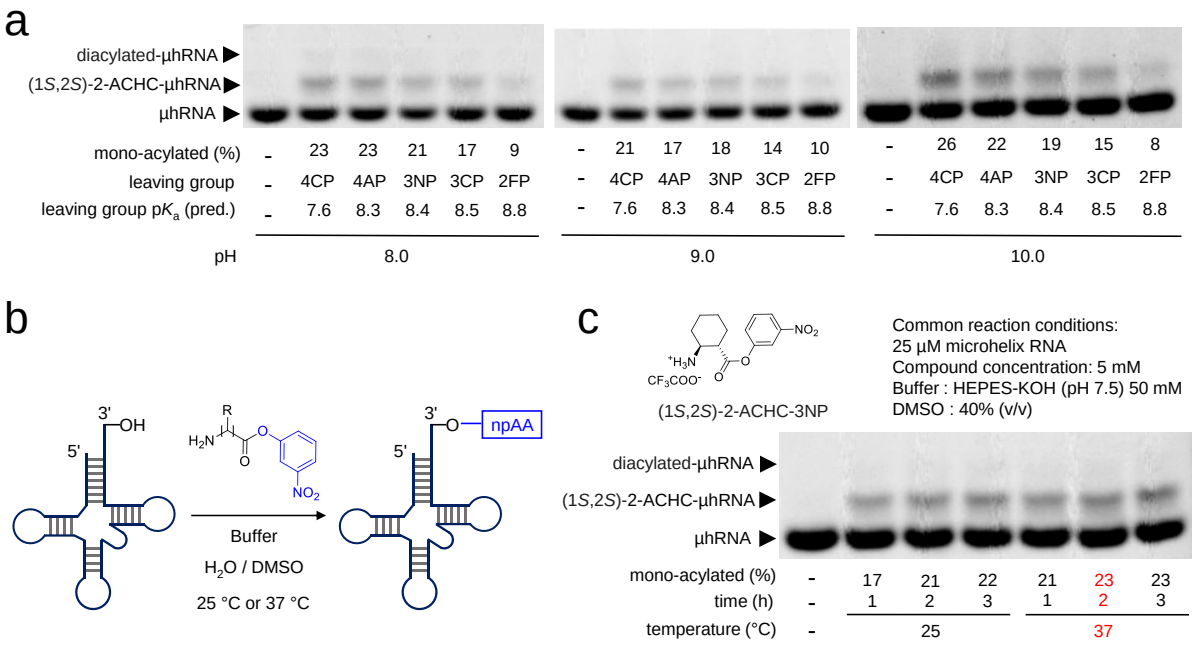
Supplementary Fig. 1: Secondary structures of a μhRNA and representative tRNAs used for acylation of nonproteinogenic amino acids. a, Secondary structure of a μhRNA (microhelix RNA) used for acylation evaluation through acid denaturing PAGE. **b,** Secondary structure of *E. coli* $tRNA^{Tyr}$ utilized in 3' terminal fragment analysis of acylated tRNAs. **c,** $tRNA^{Pro1E2}_{GGU}$ used for elongation of nonproteinogenic amino acids by decoding ACC codon. **d,** $tRNA^{iniP}$ used for initiation of nonproteinogenic amino acids at AUG codon.



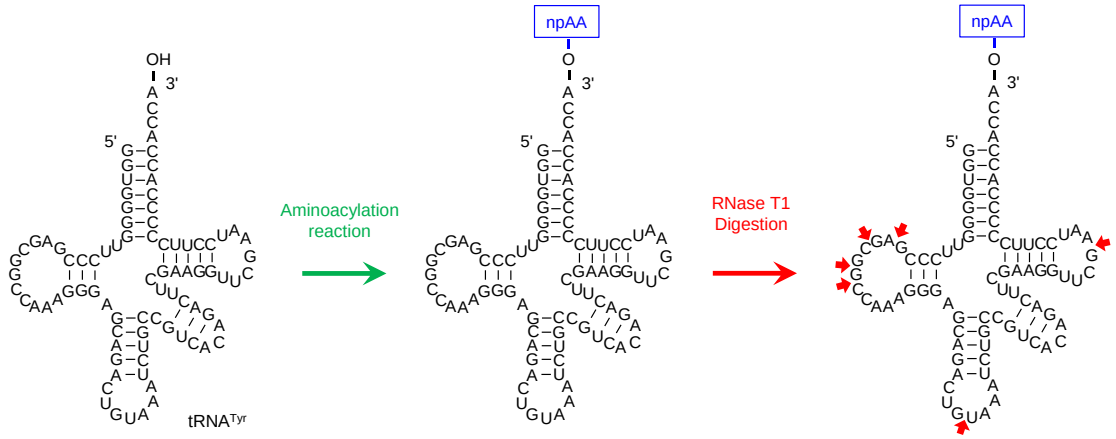
Supplementary Fig. 2: Whole gel data of Fig. 1a and Fig. 1b. a, Whole gel data of Fig. 1a. **b,** Whole gel data of Fig. 1b.



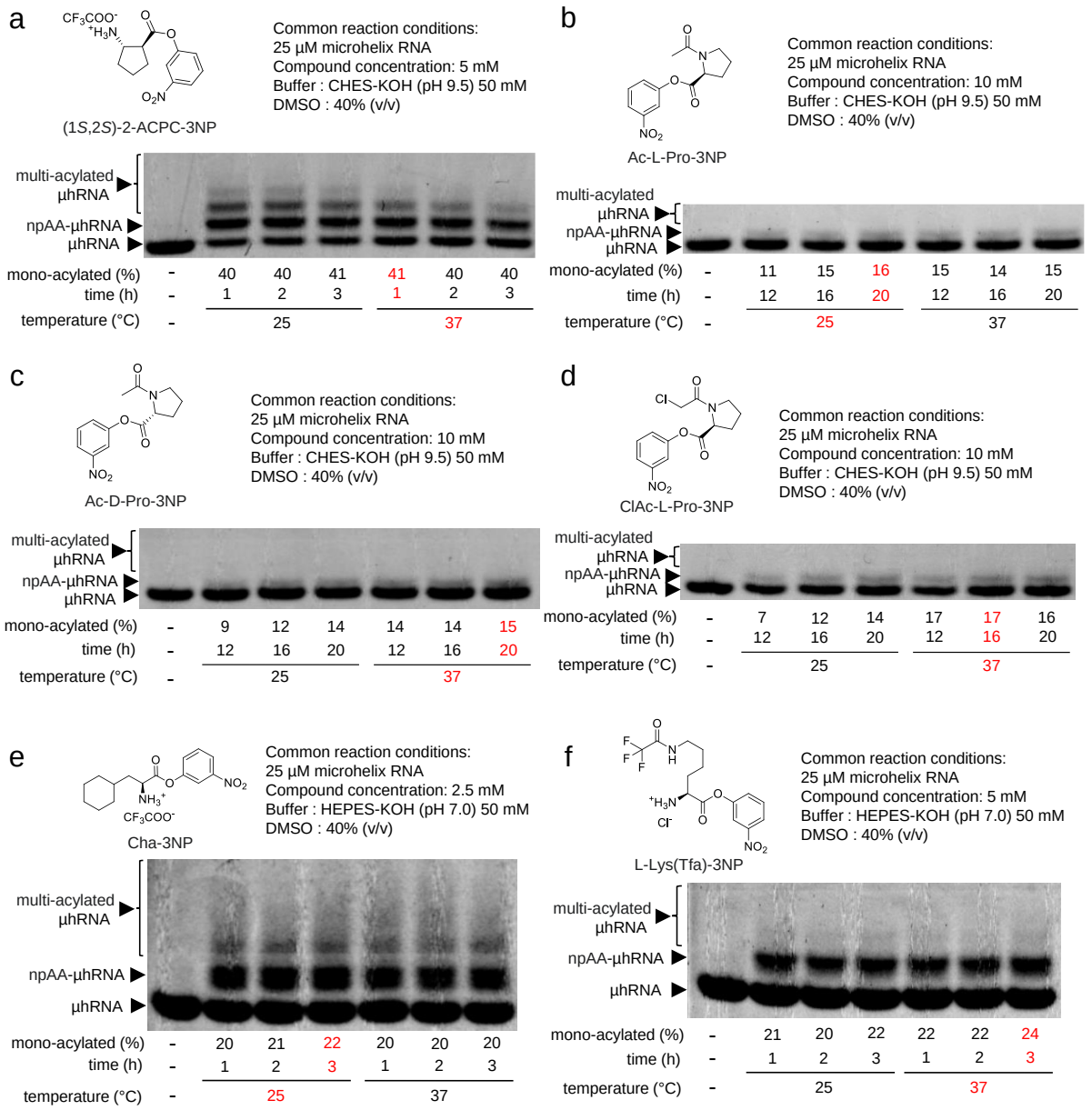
Supplementary Fig. 3: Schematic depiction of tRNA acylation patterns using NCAs for acylation. 3'-Terminal hydroxy groups are located in the outermost space of tRNA and in the single stranded region. Therefore, these groups are in the most accessible position to react with acylation reagents such as NCAs. Internal 2'-hydroxy groups are shielded by the tRNA secondary structure. After acylation using NCAs, one nonproteinogenic amino acid (npAA) can be acylated to tRNA, designated as mono-acylation. Additionally, multiple npAAs can also be acylated to tRNA, which is referred to as multi-acylation. Multi-acylation can be classified into two patterns: polymerization (multi-acylation type I) and acylation to various hydroxy groups (multi-acylation type II). Ribosomal incorporation of multi-acylated products contains uncertainty. In the case of the multi-acylation type I, there is no chance to participate in the translation events, if elongator tRNAs are used. For the multi-acylation type II, if acylation to 2'-hydroxy groups interrupts molecular recognitions, such as tRNA-translation factors interaction or tRNA-mRNA recognitions, translation events can be hindered. Therefore, acylation conditions for maximizing the mono-acylation are required for increasing the chance of successes in the translation events.



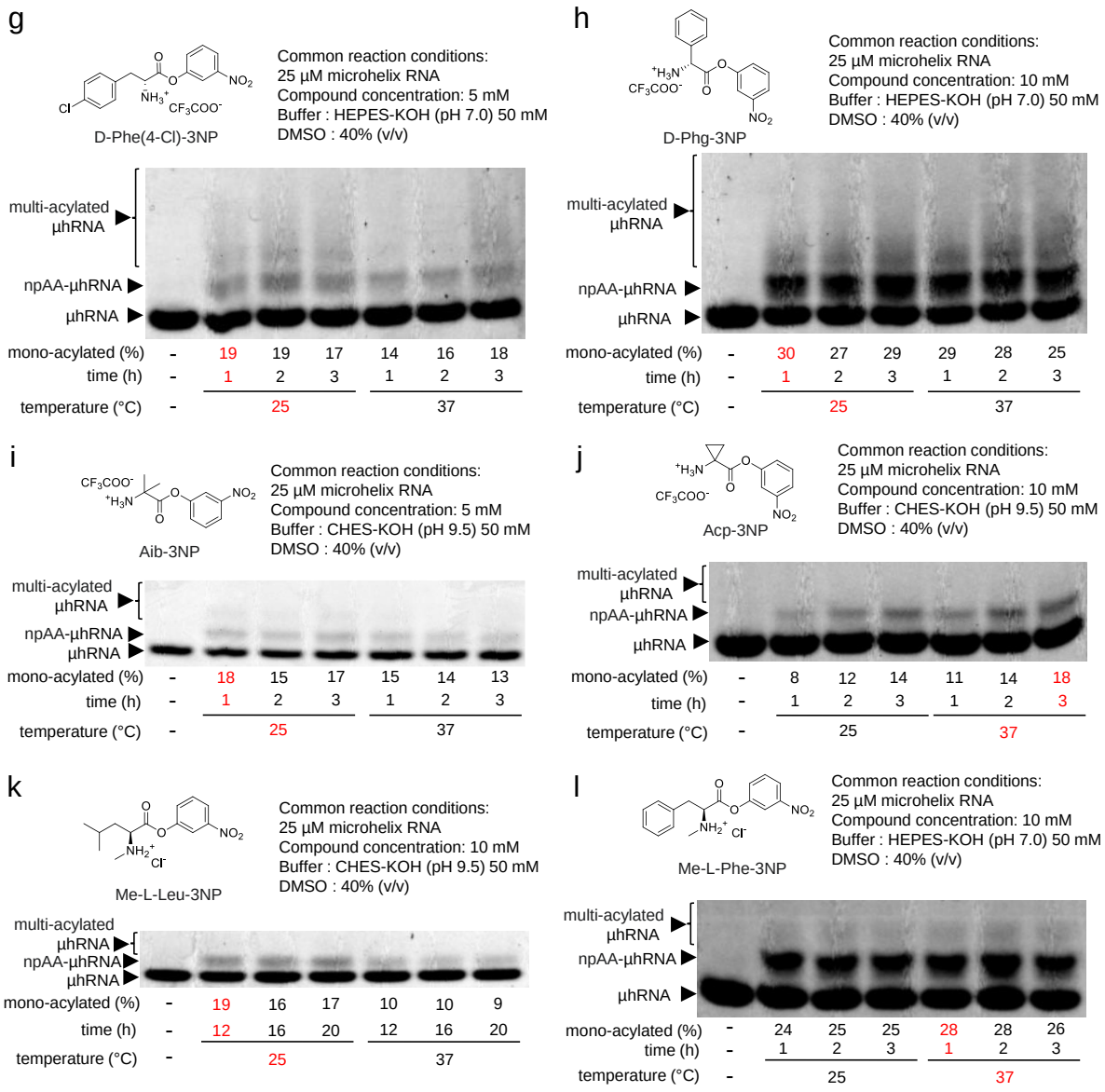
Supplementary Fig. 4: Non-enzymatic aminoacylation by phenol ester derivatives. a, Acid denaturing PAGE based pH dependent leaving group engineering experiment. **b,** Schematic representation of the non-enzymatic aminoacylation reaction. **c,** Optimization of the non-enzymatic aminoacylation reaction utilizing (1*S*,2*S*)-2-ACHC-3NP. After pre-running at 100 V for 30 minutes, samples were loaded to the gel. Further gel running was conducted at 120 V for 3 hours. 50 mM sodium acetate buffer (pH 5.0) was used for gel running. μhRNA bands were stained by ethidium bromide. Optimal conditions for acylation were highlighted by red numbers.



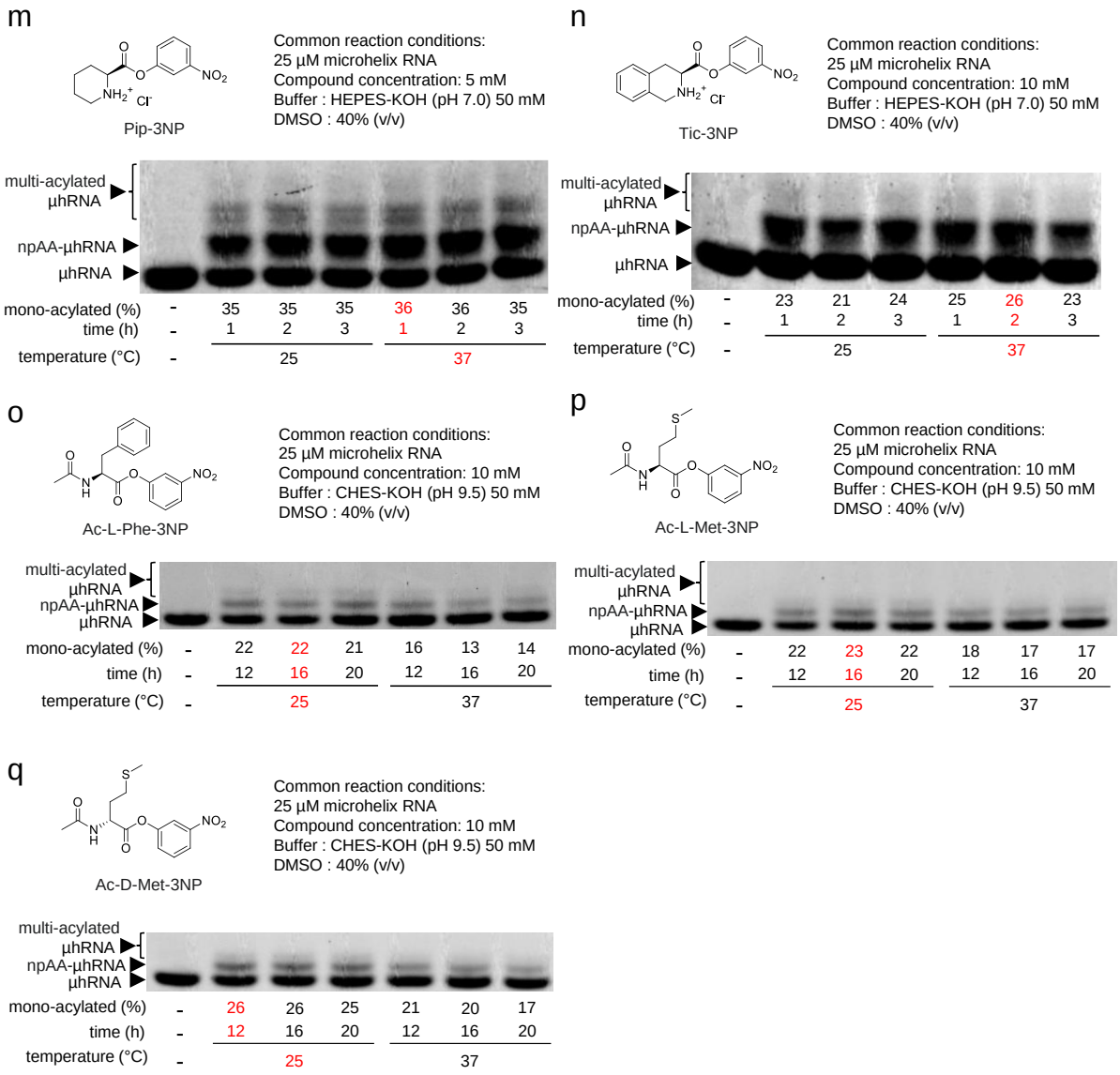
Supplementary Fig. 5: Schematic procedure of analysis of 3'-terminal fragments of aminoacyl-tRNA^{Tyr}. After aminoacylation of tRNA^{Tyr}, RNase T1 treatment was performed sequentially. Red arrows indicate the digestion points of RNase T1 in the tRNA^{Tyr}. 3'-terminal fragments after RNase T1 reaction were analyzed by MALDI-TOF MS analysis. “npAA” denotes nonproteinogenic amino acid.



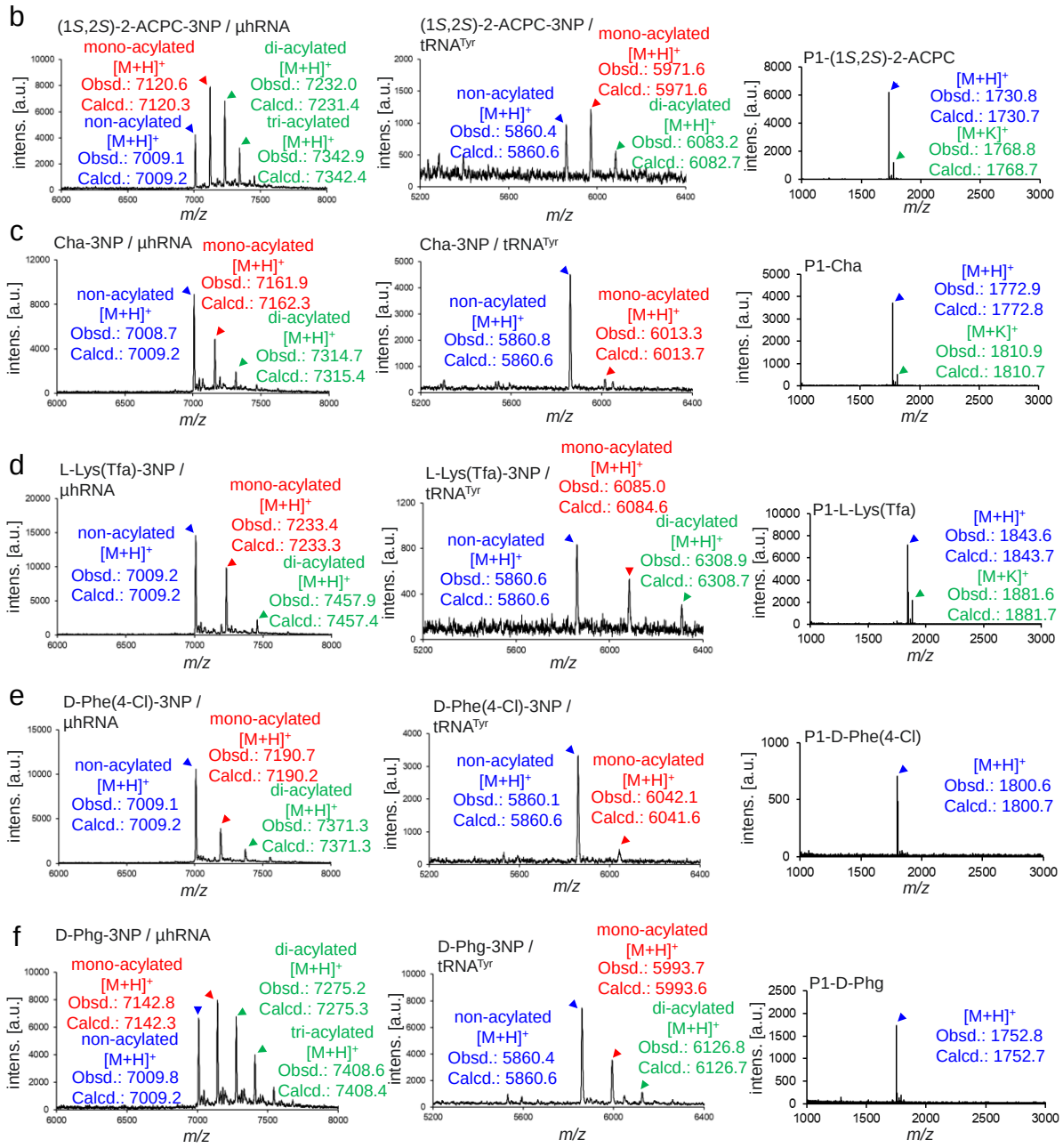
Supplementary Fig. 6: Acid denaturing PAGE analysis of aminoacyl-µhRNA acylated by non-enzymatic aminoacylation utilizing 3NP class.



Supplementary Fig. 6 continued.



Supplementary Fig. 6 continued. Non-enzymatic μhRNA acylation reaction was conducted by (1*S*,2*S*)-2-ACPC-3NP (a), Ac-L-Pro-3NP (b), Ac-D-Pro-3NP (c), ClAc-L-Pro-3NP (d), Cha-3NP (e), L-Lys(Tfa)-3NP (f), D-Phe(4-Cl)-3NP (g), D-Phg-3NP (h), Aib-3NP (i), Acp-3NP (j), Me-L-Leu-3NP (k), Me-L-Phe-3NP (l), Pip-3NP (m), Tic-3NP (n), Ac-L-Phe-3NP (o), Ac-L-Met-3NP (p), and Ac-D-Met-3NP (q). After pre-running at 100 V for 30 minutes, samples were loaded to the gel. Further gel running was conducted at 120 V for 3 hours. 50 mM sodium acetate buffer (pH 5.0) was used for gel running. μhRNA bands were stained by ethidium bromide. Optimal conditions for acylation were highlighted by red numbers.



Supplementary Fig. 7: MALDI-TOF MS-based evaluation of non-enzymatic aminoacylation utilizing 3NP class.

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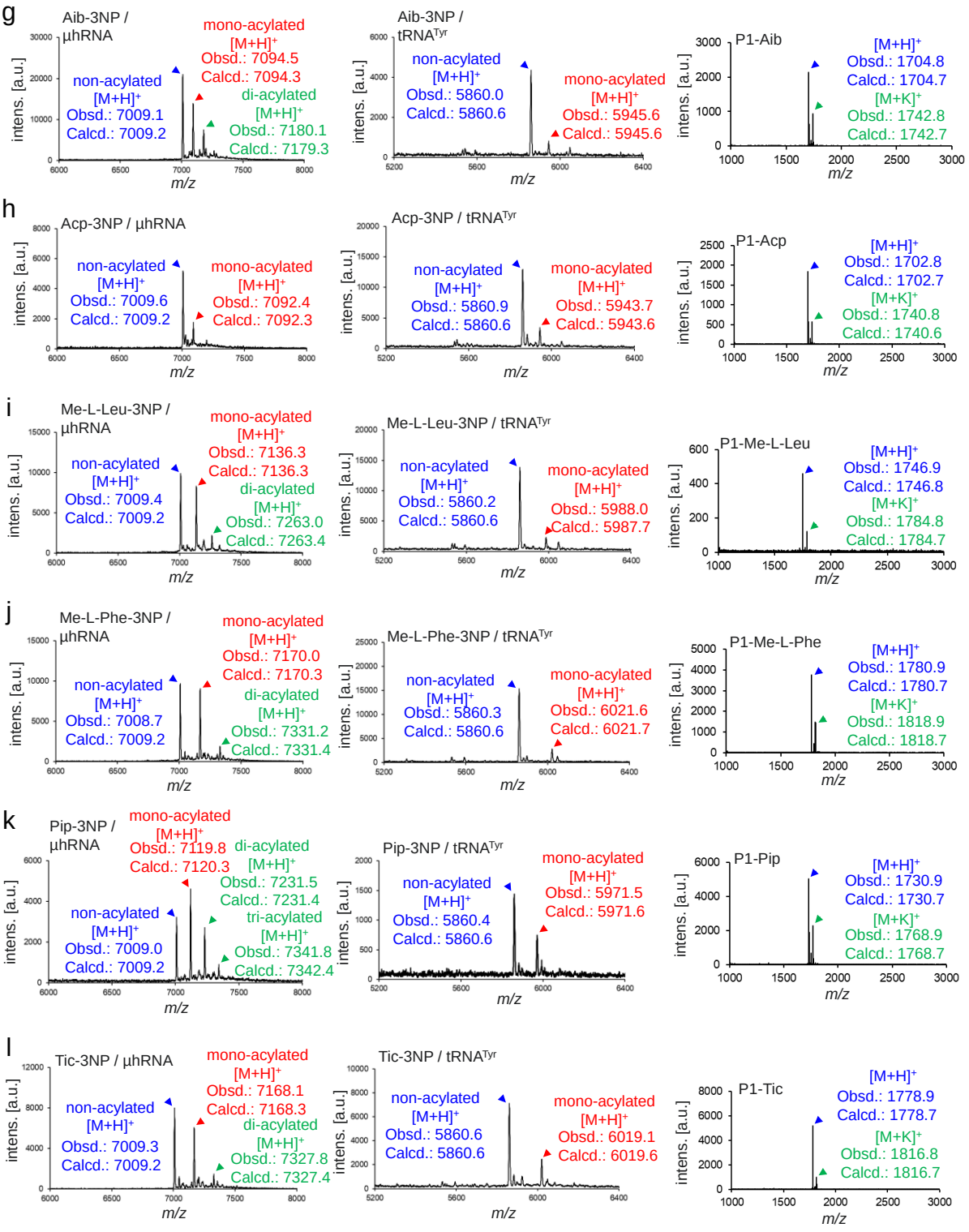

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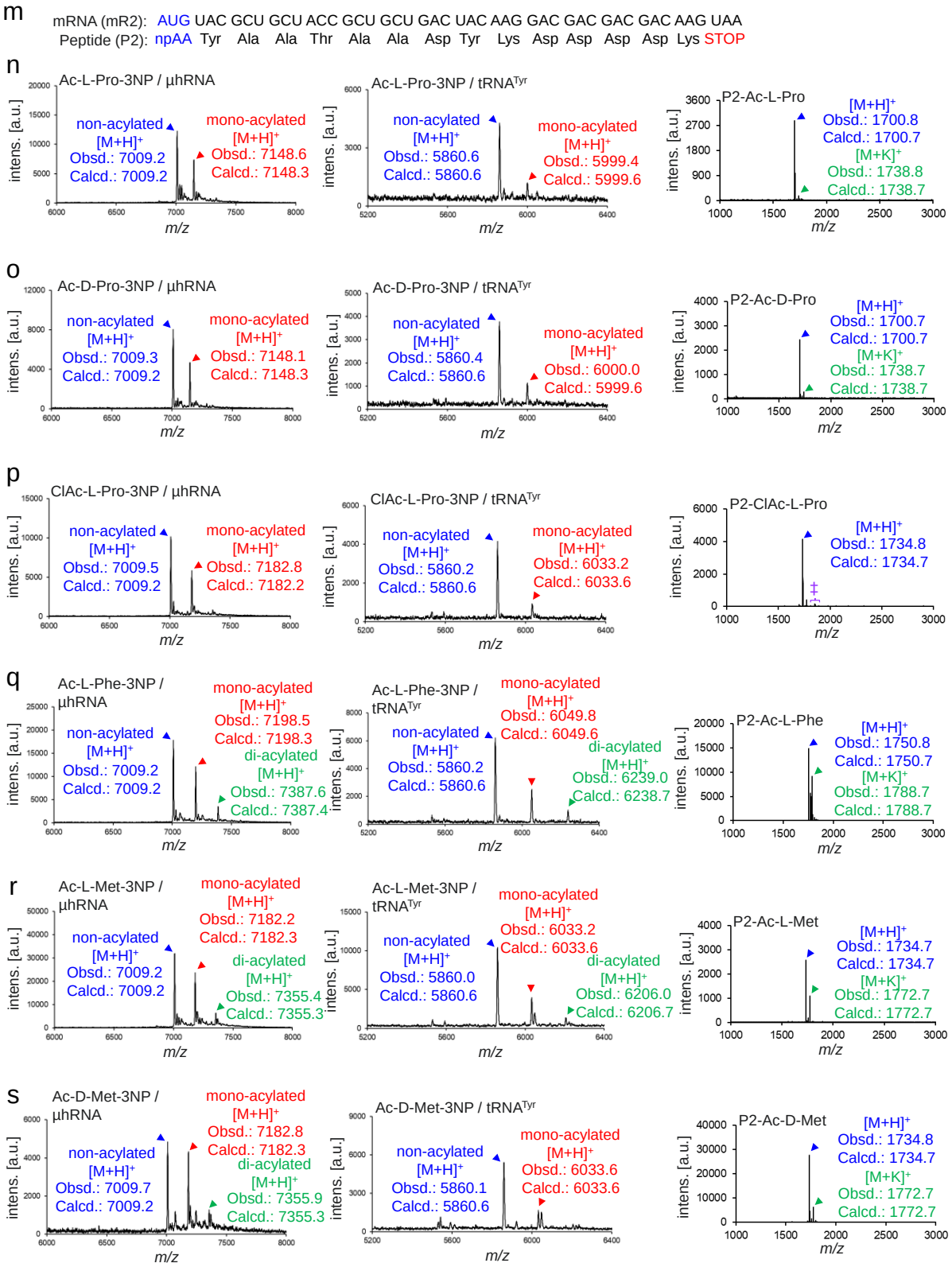
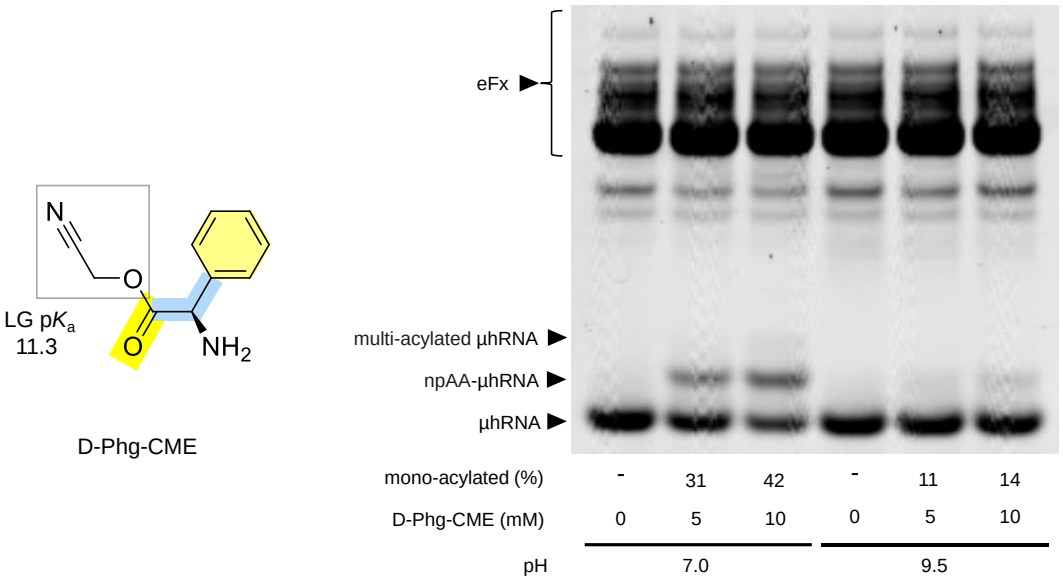
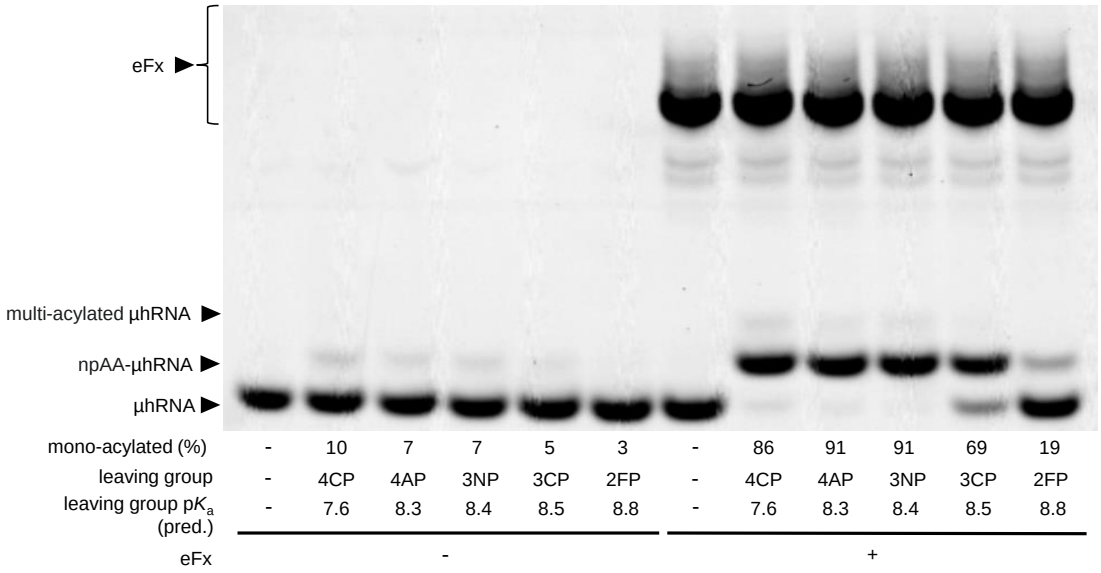


Supplementary Fig. 7 continued.

Supplementary Fig. 7 continued. μhRNA and tRNAs were acylated by non-enzymatic aminoacylation method using 3NP class compounds. After aminoacylation, npAA-μhRNAs were directly detected by MALDI-TOF MS. npAA-$tRNA^{Tyr}$s were treated with RNase T1, and digested 3'-terminal fragments were analyzed by MALDI-TOF MS. For the model peptide synthesis, npAA-$tRNA^{Pro1E2}_{GGU}$s were used for ribosomal elongation of npAAs. The mRNA (mR1) sequence for evaluation of npAA elongation and the corresponding peptide (P1) sequence are presented in (a). MALDI-TOF MS spectra of abovementioned experiments using (1*S*,2*S*)-2-ACPC-3NP (b), Cha-3NP (c), L-Lys(Tfa)-3NP (d), D-Phe(4-Cl)-3NP (e), D-Phg-3NP (f), Aib-3NP (g), Acp-3NP (h), Me-L-Leu-3NP (i), Me-L-Phe-3NP (j), Pip-3NP (k) and Tic-3NP (l) are presented with npAA elongation results, respectively. For the ribosomal initiation of npAAs, npAA-$tRNA^{iniP}$s acylated by the non-enzymatic method were used. The mRNA (mR2) sequence for evaluation of npAA initiation and the corresponding peptide (P2) sequence are presented in (m). MALDI-TOF MS spectra of the evaluation experiments utilizing Ac-L-Pro-3NP (n), Ac-D-Pro-3NP (o), ClAc-L-Pro-3NP (p), Ac-L-Phe-3NP (q), Ac-L-Met-3NP (r) and Ac-D-Met-3NP (s) are presented, respectively. “Obsd.” and “Calcd.” denote observed and calculated m/z values, respectively. Double dagger indicates DTT conjugated translated peptide.

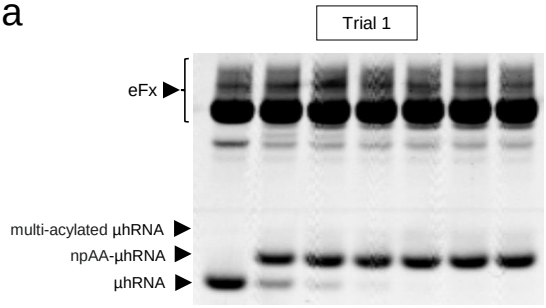


Supplementary Fig. 8: Flexizyme catalysis evaluation of D-Phg-CME. µhRNA acylation reaction was conducted by standard Flexizyme reaction in the presence of eFx. Reaction mixtures containing 25 µM eFx, 25 µM microhelix RNA, 0-10 mM D-Phg-CME, 600 mM $MgCl_2$, DMSO 20% (v/v) and 50 mM pH buffer (pH 7.0 HEPES-KOH or pH 9.5 CHES-KOH for respective pH) were equally incubated on ice for 16 hours. After gel pre-running at 100 V for 30 minutes, samples were loaded to the acid denaturing PAGE gel. Further gel running was conducted at 120 V for 3 hours. 50 mM sodium acetate buffer (pH 5.0) was used for gel running. µhRNA bands were stained by ethidium bromide.

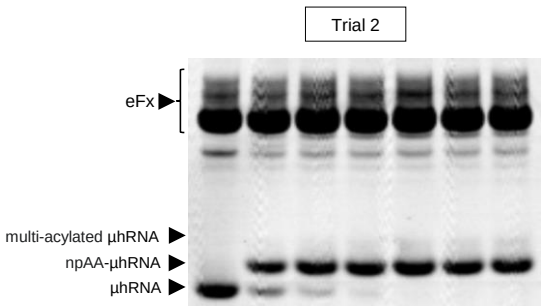


Supplementary Fig. 9: Whole gel data of Fig. 3b. Phenol ester derivatives were tested for eFx catalysis evaluation. Reaction mixtures containing 25 µM µhRNA, 50 mM HEPES-KOH (pH 7.0), 600 mM $MgCl_2$ and DMSO 20% (v/v) were equally incubated on ice for 2 hours. For the compound treated samples, 5 mM activated amino acids were added. For eFx incubated samples, 25 µM eFx was added.

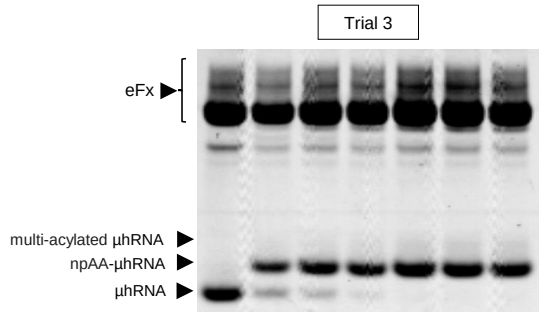
a



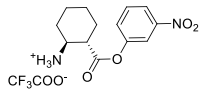
mono-acylated (%)	-	66	80	85	85	86	87
Time (min)	-	10	20	30	60	90	120



mono-acylated (%)	-	68	81	86	88	90	88
Time (min)	-	10	20	30	60	90	120



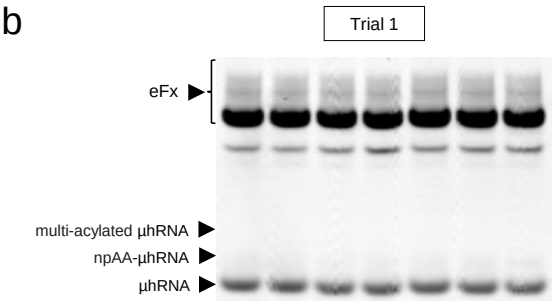
mono-acylated (%)	-	68	79	86	88	87	88
Time (min)	-	10	20	30	60	90	120



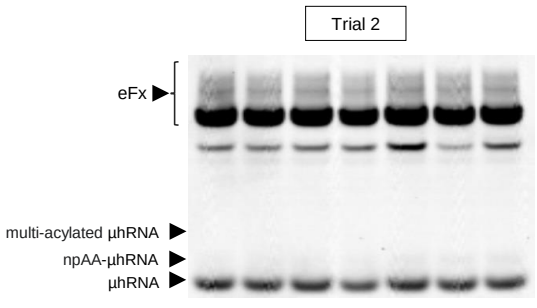
(1*S*,2*S*)-2-ACHC-3NP

Common acylation conditions :
25 μM microhelix RNA, 5 mM (1*S*,2*S*)-2-ACHC-3NP, 25 μM eFx, 600 mM $MgCl_2$, 50 mM HEPES-KOH (pH 7.0), DMSO 20% (v/v), on ice, 10 min – 120 min

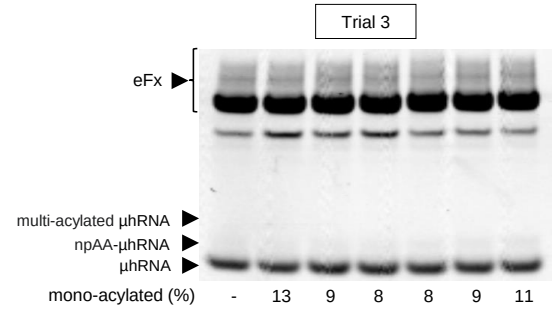
b



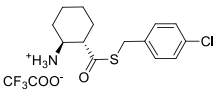
mono-acylated (%)	-	14	7	8	10	10	10
Time (min)	-	10	20	30	60	90	120



mono-acylated (%)	-	11	10	9	10	9	11
Time (min)	-	10	20	30	60	90	120



mono-acylated (%)	-	13	9	8	8	9	11
Time (min)	-	10	20	30	60	90	120

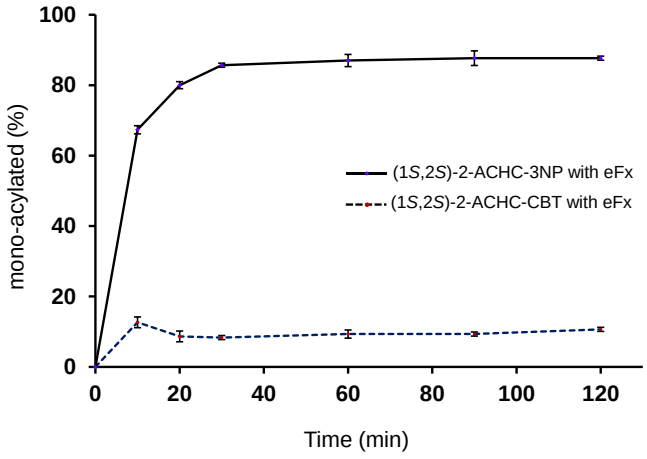


(1*S*,2*S*)-2-ACHC-CBT

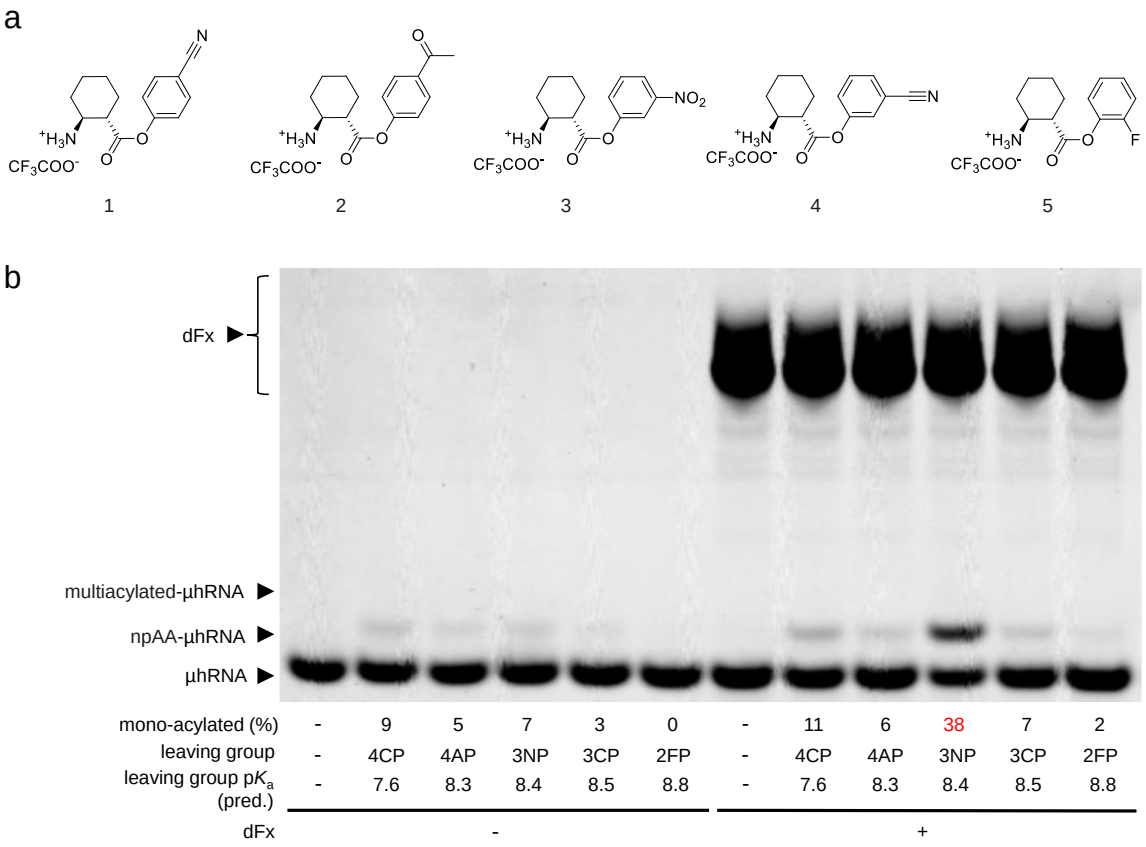
Common acylation conditions :
25 μM microhelix RNA, 5 mM (1*S*,2*S*)-2-ACHC-CBT, 25 μM eFx, 600 mM $MgCl_2$, 50 mM HEPES-KOH (pH 7.0), DMSO 20% (v/v), on ice, 10 min – 120 min

Supplementary Fig. 10: Time course aminoacylation utilizing 3NP class and CBT class.

C

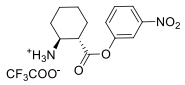


Supplementary Fig. 10 continued. a-b, μhRNA acylation reaction was conducted based on the standard flexizyme reaction in the presence of eFx. Reaction mixtures containing 25 μM eFx, 25 μM microhelix RNA, 5 mM activated amino acid, 600 mM $MgCl_2$, DMSO 20% (v/v) and 50 mM HEPES-KOH (pH 7.0) were incubated on ice with various time points. For the activated amino acids, (1*S*,2*S*)-2-ACHC-3NP was used in (a). (1*S*,2*S*)-2-ACHC-CBT was tested in (b). After gel pre-running at 100 V for 30 minutes, samples were loaded to the acid denaturing PAGE gel. Further gel running was conducted at 120 V for 3 hours. 50 mM sodium acetate buffer (pH 5.0) was used for gel running. μhRNA bands were stained by ethidium bromide. **c,** Graphical presentation of monoacylation yields over time, based on acid denaturing PAGE data as shown in (a) and (b); n = 3, error bars; s.d.



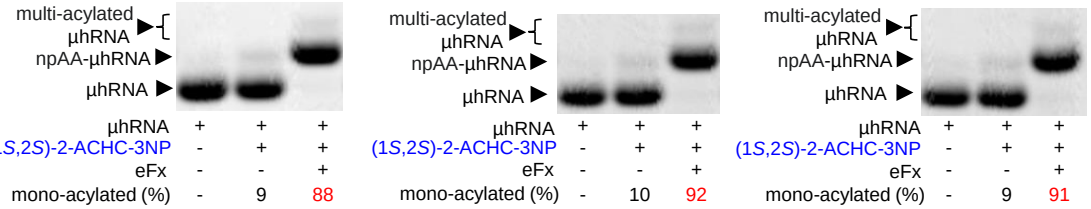
Supplementary Fig. 11: Flexizyme catalysis evaluation of phenol ester derivatives with dFx. a, Structures of activated amino acids for flexizyme catalysis evaluation. **b,** Acid denaturing PAGE gel data. Phenol ester derivatives were tested for dFx catalysis evaluation. Reaction mixtures containing 25 μM microhelix RNA, 50 mM HEPES-KOH (pH 7.0), 20 mM $MgCl_2$ and DMSO 20% (v/v) were equally incubated on ice for 2 hours. For the compound treated samples, 5 mM activated amino acids were added. For dFx incubated samples, 25 μM dFx was added. After gel pre-running at 100 V for 30 minutes, samples were loaded to the acid denaturing PAGE gel. Further gel running was conducted at 120 V for 3 hours. 50 mM sodium acetate buffer (pH 5.0) was used for gel running. μhRNA bands were stained by ethidium bromide.

a



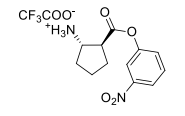
(1*S*,2*S*)-2-ACHC-3NP

Common reaction conditions :
25 μM microhelix RNA, 600 mM $MgCl_2$, HEPES-KOH (pH 7.0) 50 mM,
DMSO 20% (v/v), on ice, 2 h
For (1*S*,2*S*)-2-ACHC-3NP (+) samples, 5 mM of (1*S*,2*S*)-2-ACHC-3NP was added
For eFx (+) sample, 25 μM of eFx was added



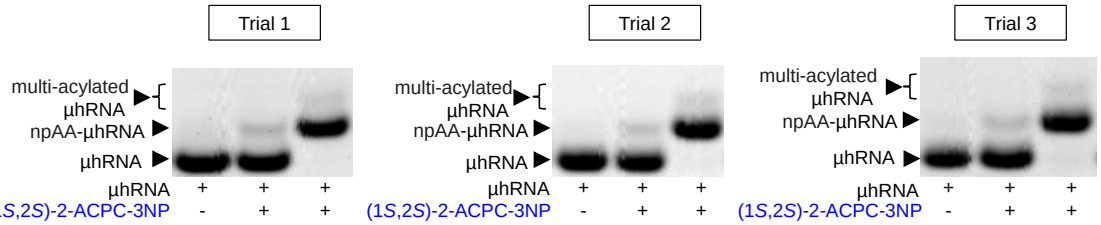
	Trial 1			Trial 2			Trial 3		
μhRNA	+	+	+	+	+	+	+	+	+
(1*S*,2*S*)-2-ACHC-3NP	-	+	+	-	+	+	-	+	+
eFx	-	-	+	-	-	+	-	-	+
mono-acylated (%)	-	9	88	-	10	92	-	9	91

b



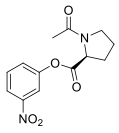
(1*S*,2*S*)-2-ACPC-3NP

Common reaction conditions :
25 μM microhelix RNA, 600 mM $MgCl_2$, HEPES-KOH (pH 7.0) 50 mM,
DMSO 20% (v/v), on ice, 2 h
For (1*S*,2*S*)-2-ACPC-3NP (+) samples, 5 mM of (1*S*,2*S*)-2-ACPC-3NP was added
For eFx (+) sample, 25 μM of eFx was added



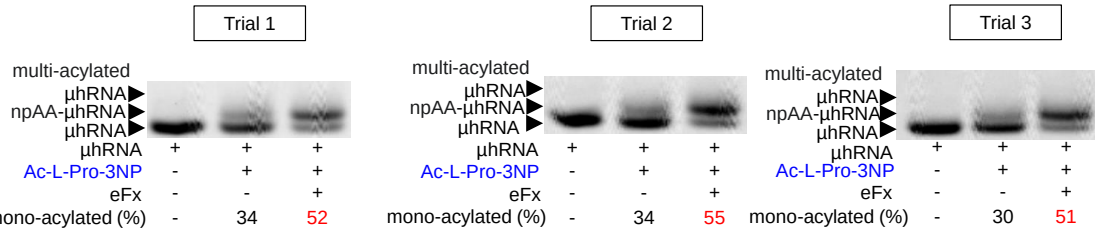
	Trial 1			Trial 2			Trial 3		
μhRNA	+	+	+	+	+	+	+	+	+
(1*S*,2*S*)-2-ACPC-3NP	-	+	+	-	+	+	-	+	+
eFx	-	-	+	-	-	+	-	-	+
mono-acylated (%)	-	13	88	-	12	89	-	13	90

c



Ac-L-Pro-3NP

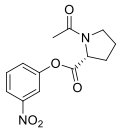
Common reaction conditions :
25 μM microhelix RNA, 600 mM $MgCl_2$, CHES-KOH (pH 9.5) 50 mM,
DMSO 20% (v/v), on ice, 16 h
For Ac-L-Pro-3NP (+) samples, 10 mM of Ac-L-Pro-3NP was added
For eFx (+) sample, 25 μM of eFx was added



	Trial 1			Trial 2			Trial 3		
μhRNA	+	+	+	+	+	+	+	+	+
Ac-L-Pro-3NP	-	+	+	-	+	+	-	+	+
eFx	-	-	+	-	-	+	-	-	+
mono-acylated (%)	-	34	52	-	34	55	-	30	51

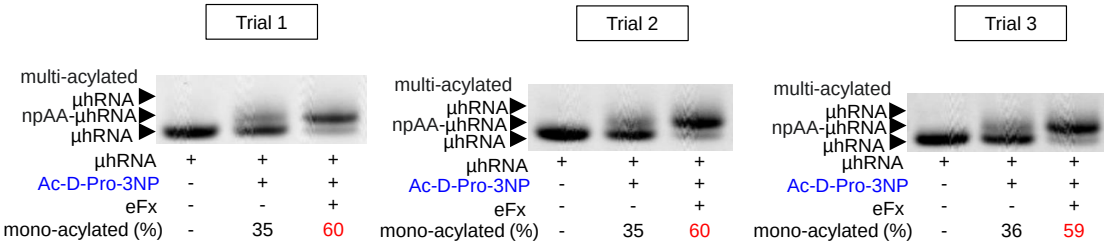
Supplementary Fig. 12: Flexizyme catalysis evaluation of structurally diverse 3NP class.

d



Ac-D-Pro-3NP

Common reaction conditions :
25 μM microhelix RNA, 600 mM $MgCl_2$, CHES-KOH (pH 9.5) 50 mM,
DMSO 20% (v/v), on ice, 16 h
For Ac-D-Pro-3NP (+) samples, 10 mM of Ac-D-Pro-3NP was added
For eFx (+) sample, 25 μM of eFx was added



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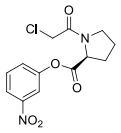
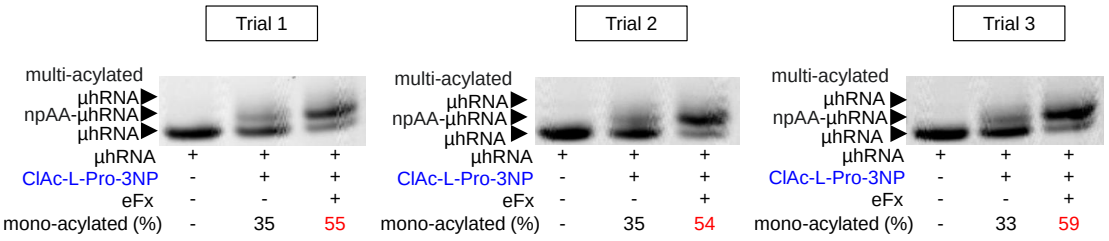


ClAc-L-Pro-3NP

Common reaction conditions :
25 μM microhelix RNA, 600 mM $MgCl_2$, CHES-KOH (pH 9.5) 50 mM,
DMSO 20% (v/v), on ice, 16 h
For ClAc-L-Pro-3NP (+) samples, 10 mM of ClAc-L-Pro-3NP was added
For eFx (+) sample, 25 μM of eFx was added



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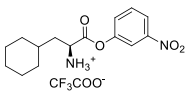
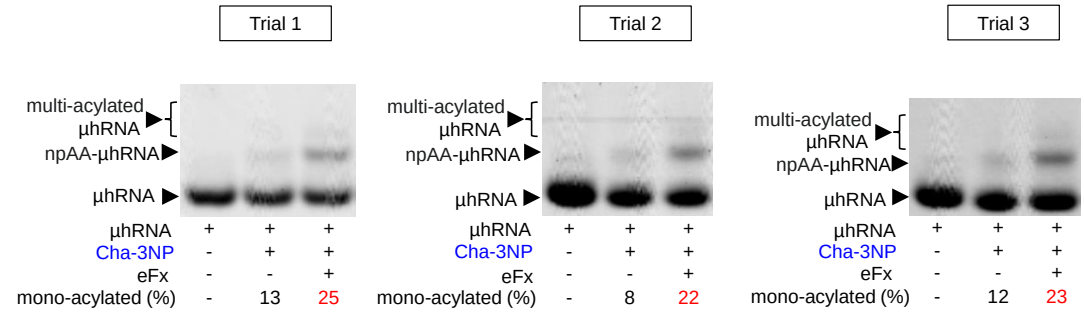


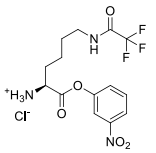
Cha-3NP

Common reaction conditions :
25 μM microhelix RNA, 600 mM $MgCl_2$, HEPES-KOH (pH 7.0) 50 mM,
DMSO 20% (v/v), on ice, 2 h
For Cha-3NP (+) samples, 5 mM of Cha-3NP was added
For eFx (+) sample, 25 μM of eFx was added



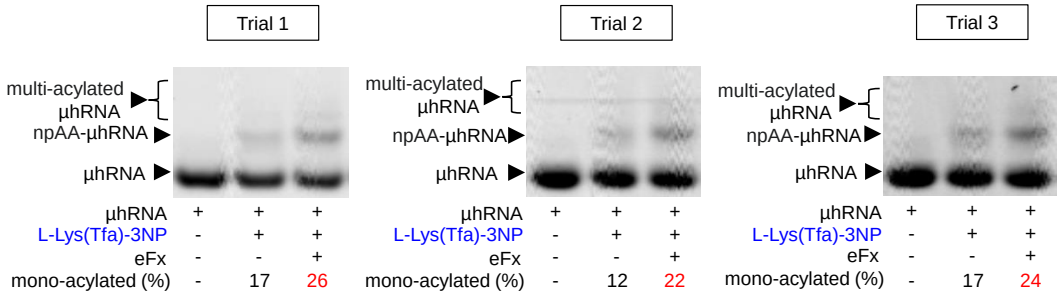
Supplementary Fig. 12 continued.

g



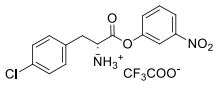
L-Lys(Tfa)-3NP

Common reaction conditions :
25 μM microhelix RNA, 600 mM $MgCl_2$, HEPES-KOH (pH 7.0) 50 mM, DMSO 20% (v/v), on ice, 2 h
For L-Lys(Tfa)-3NP (+) samples, 5 mM of L-Lys(Tfa)-3NP was added
For eFx (+) sample, 25 μM of eFx was added



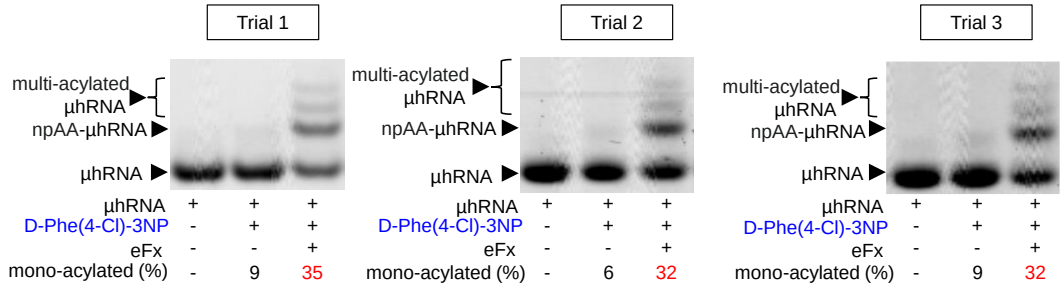
	Trial 1			Trial 2			Trial 3		
μhRNA	+	+	+	+	+	+	+	+	+
L-Lys(Tfa)-3NP	-	+	+	-	+	+	-	+	+
eFx	-	-	+	-	-	+	-	-	+
mono-acylated (%)	-	17	26	-	12	22	-	17	24

h



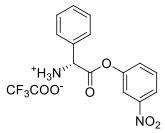
D-Phe(4-Cl)-3NP

Common reaction conditions :
25 μM microhelix RNA, 600 mM $MgCl_2$, HEPES-KOH (pH 7.0) 50 mM, DMSO 20% (v/v), on ice, 2 h
For D-Phe(4-Cl)-3NP (+) samples, 5 mM of D-Phe(4-Cl)-3NP was added
For eFx (+) sample, 25 μM of eFx was added



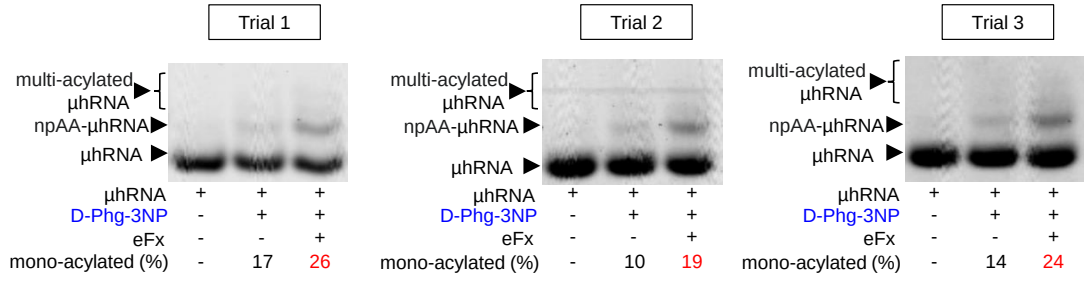
	Trial 1			Trial 2			Trial 3		
μhRNA	+	+	+	+	+	+	+	+	+
D-Phe(4-Cl)-3NP	-	+	+	-	+	+	-	+	+
eFx	-	-	+	-	-	+	-	-	+
mono-acylated (%)	-	9	35	-	6	32	-	9	32

i



D-Phg-3NP

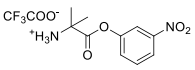
Common reaction conditions :
25 μM microhelix RNA, 600 mM $MgCl_2$, HEPES-KOH (pH 7.0) 50 mM, DMSO 20% (v/v), on ice, 2 h
For D-Phg-3NP (+) samples, 5 mM of D-Phg-3NP was added
For eFx (+) sample, 25 μM of eFx was added



	Trial 1			Trial 2			Trial 3		
μhRNA	+	+	+	+	+	+	+	+	+
D-Phg-3NP	-	+	+	-	+	+	-	+	+
eFx	-	-	+	-	-	+	-	-	+
mono-acylated (%)	-	17	26	-	10	19	-	14	24

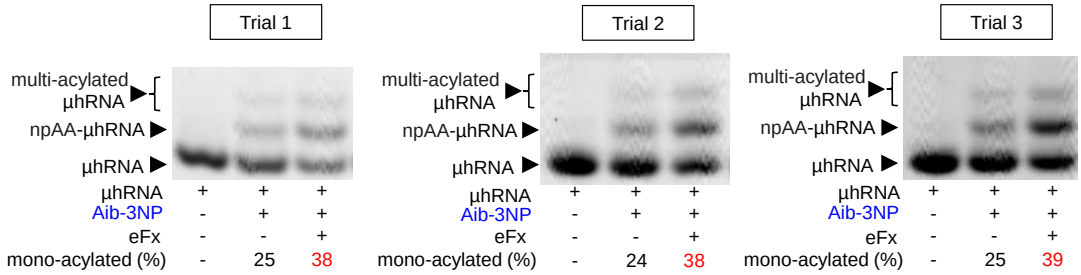
Supplementary Fig. 12 continued.

j



Aib-3NP

Common reaction conditions :
25 μM microhelix RNA, 600 mM $MgCl_2$, HEPES-KOH (pH 7.0) 50 mM, DMSO 20% (v/v), on ice, 2 h
For Aib-3NP (+) samples, 5 mM of Aib-3NP was added
For eFx (+) sample, 25 μM of eFx was added



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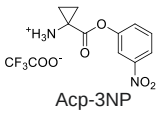
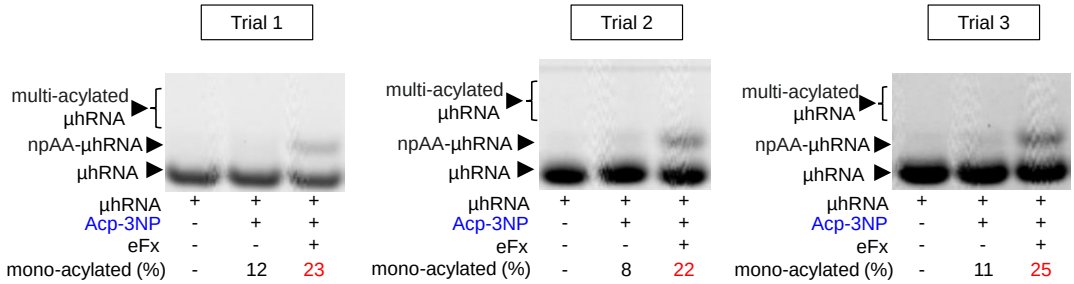


Acp-3NP

Common reaction conditions :
25 μM microhelix RNA, 600 mM $MgCl_2$, HEPES-KOH (pH 7.0) 50 mM, DMSO 20% (v/v), on ice, 2 h
For Acp-3NP (+) samples, 5 mM of Acp-3NP was added
For eFx (+) sample, 25 μM of eFx was added



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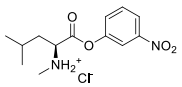
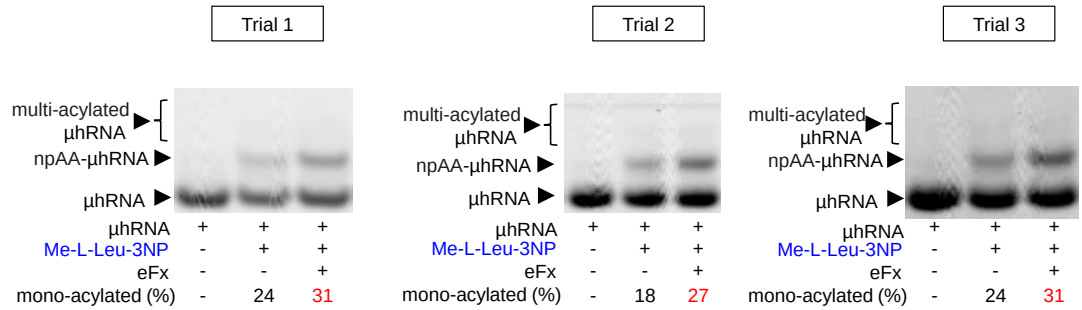


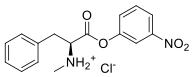
Me-L-Leu-3NP

Common reaction conditions :
25 μM microhelix RNA, 600 mM $MgCl_2$, HEPES-KOH (pH 7.0) 50 mM, DMSO 20% (v/v), on ice, 2 h
For Me-L-Leu-3NP (+) samples, 5 mM of Me-L-Leu-3NP was added
For eFx (+) sample, 25 μM of eFx was added



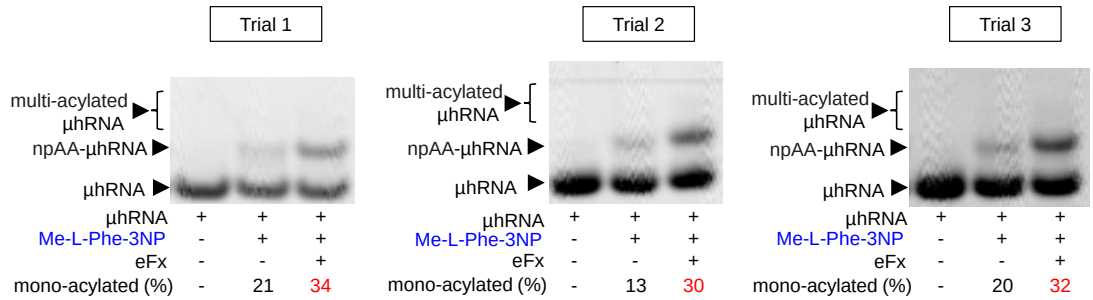
Supplementary Fig. 12 continued.

m



Me-L-Phe-3NP

Common reaction conditions :
25 μM microhelix RNA, 600 mM $MgCl_2$, HEPES-KOH (pH 7.0) 50 mM, DMSO 20% (v/v), on ice, 2 h
For Me-L-Phe-3NP(+) samples, 5 mM of Me-L-Phe-3NP was added
For eFx (+) sample, 25 μM of eFx was added



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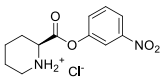
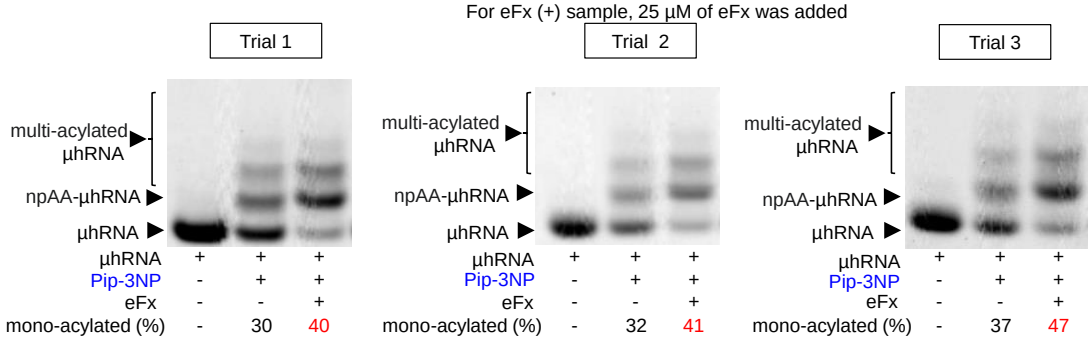
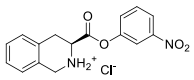


Pip-3NP

Common reaction conditions :
25 μM microhelix RNA, 600 mM $MgCl_2$, HEPES-KOH (pH 7.0) 50 mM, DMSO 20% (v/v), on ice, 2 h
For Pip-3NP (+) samples, 5 mM of Pip-3NP was added
For eFx (+) sample, 25 μM of eFx was added

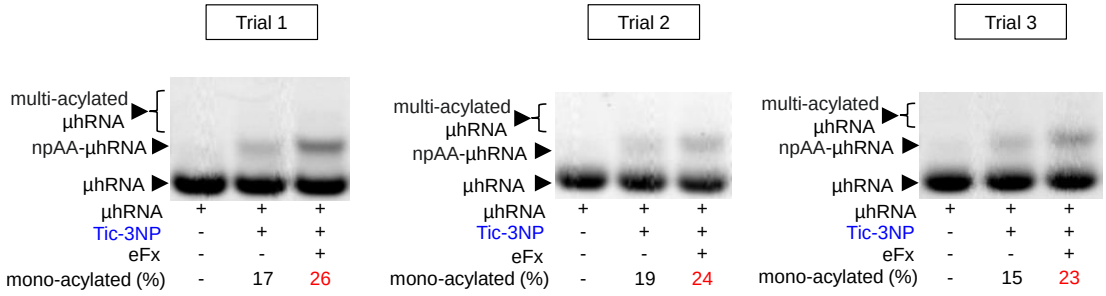


o



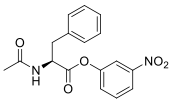
Tic-3NP

Common reaction conditions :
25 μM microhelix RNA, 600 mM $MgCl_2$, HEPES-KOH (pH 7.0) 50 mM, DMSO 20% (v/v), on ice, 2 h
For Tic-3NP (+) samples, 5 mM of Tic-3NP was added
For eFx (+) sample, 25 μM of eFx was added



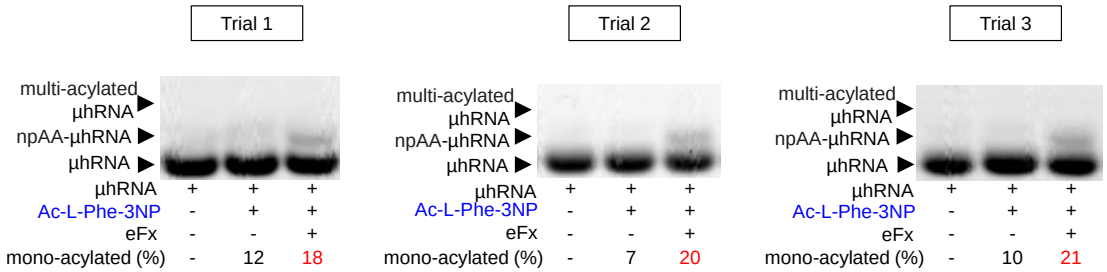
Supplementary Fig. 12 continued.

p



Ac-L-Phe-3NP

Common reaction conditions :
25 μM microhelix RNA, 600 mM $MgCl_2$, HEPES-KOH (pH 7.0) 50 mM, DMSO 20% (v/v), on ice, 2 h
For Ac-L-Phe-3NP (+) samples, 5 mM of Ac-L-Phe-3NP was added
For eFx (+) sample, 25 μM of eFx was added



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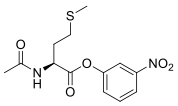
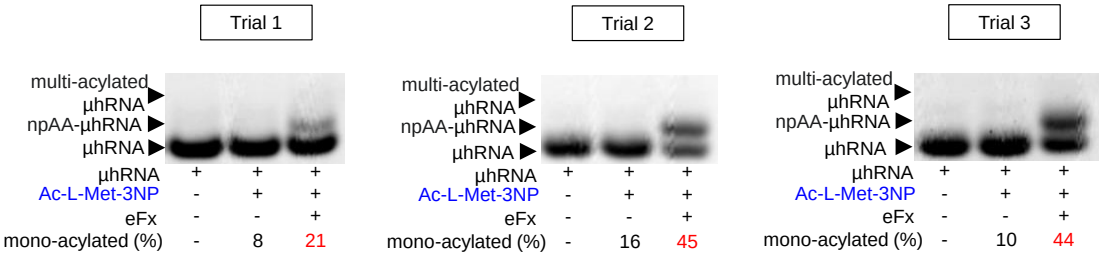


Ac-L-Met-3NP

Common reaction conditions :
25 μM microhelix RNA, 600 mM $MgCl_2$, HEPES-KOH (pH 7.0) 50 mM, DMSO 20% (v/v), on ice, 2 h
For Ac-L-Met-3NP (+) samples, 5 mM of Ac-L-Met-3NP was added
For eFx (+) sample, 25 μM of eFx was added



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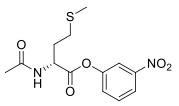
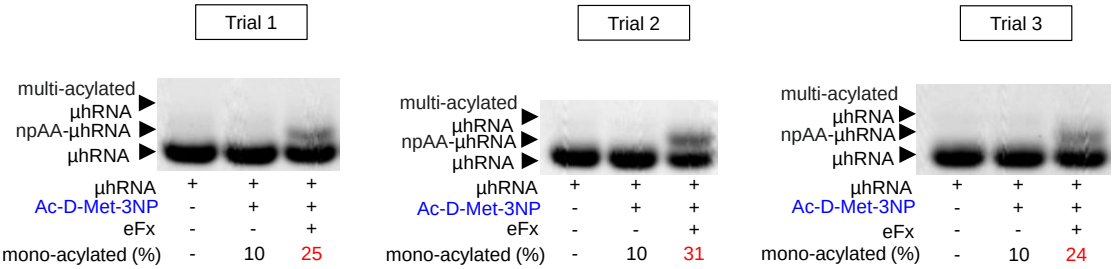
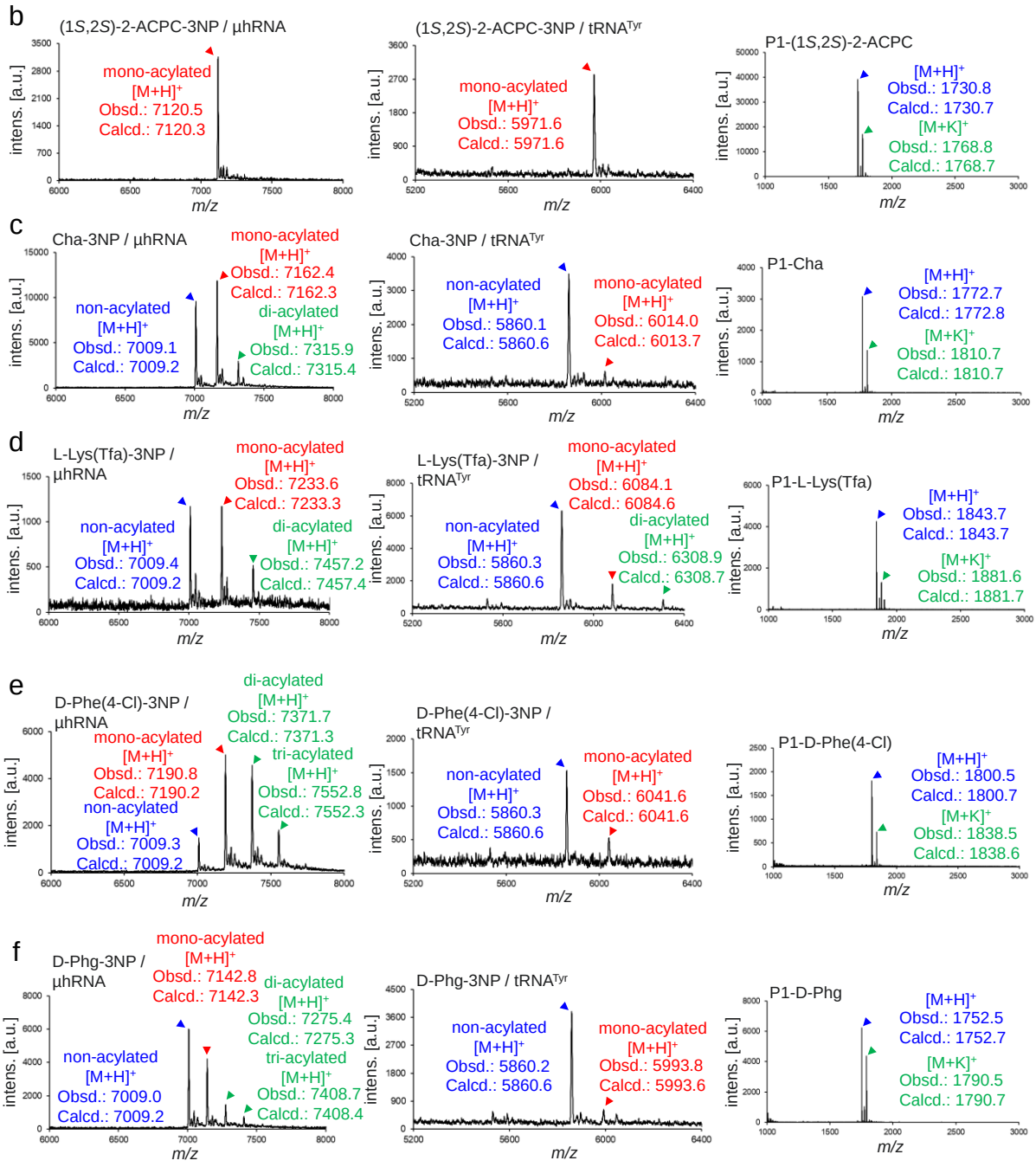


Ac-D-Met-3NP

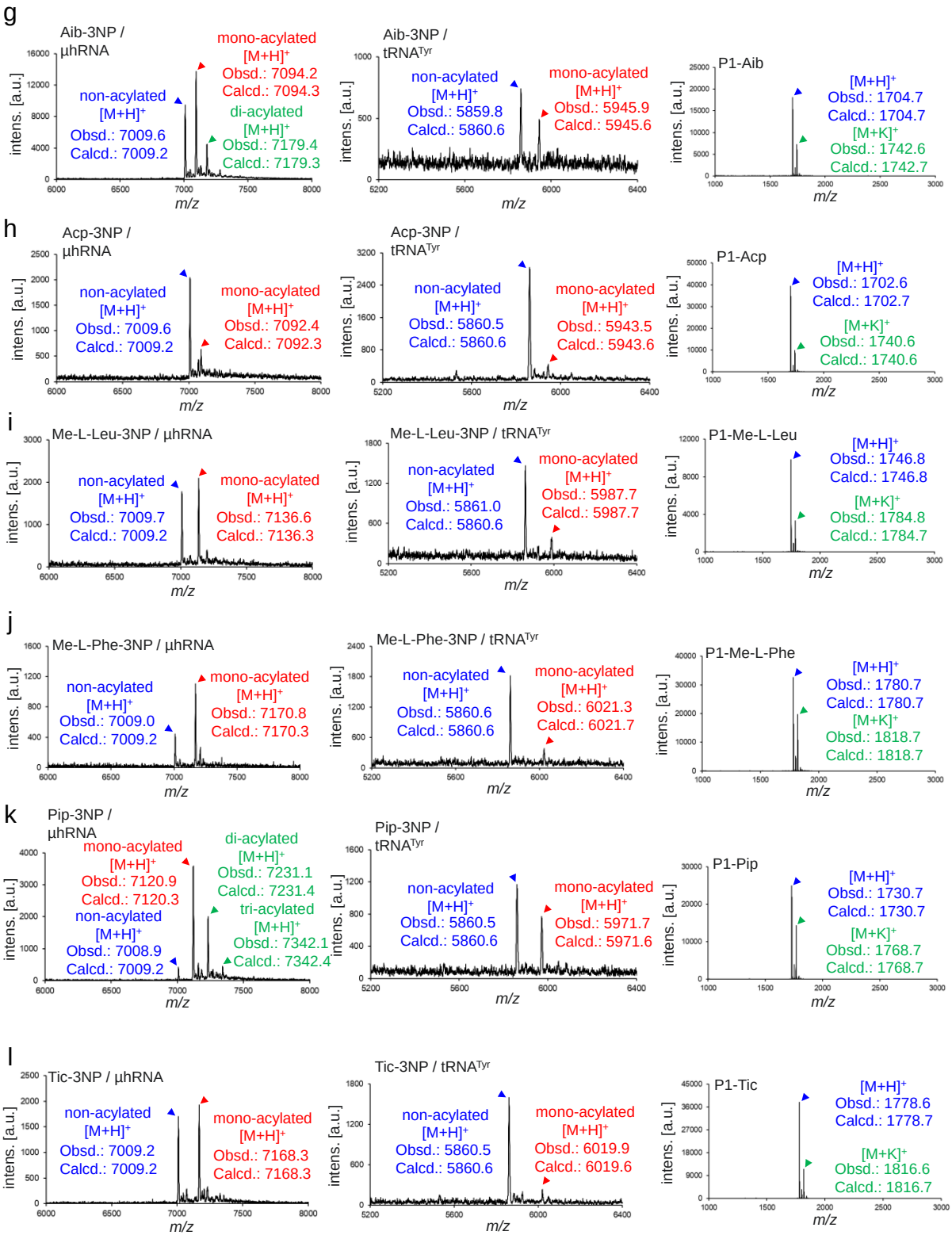
Common reaction conditions :
25 μM microhelix RNA, 600 mM $MgCl_2$, HEPES-KOH (pH 7.0) 50 mM, DMSO 20% (v/v), on ice, 2 h
For Ac-D-Met-3NP (+) samples, 5 mM of Ac-D-Met-3NP was added
For eFx (+) sample, 25 μM of eFx was added



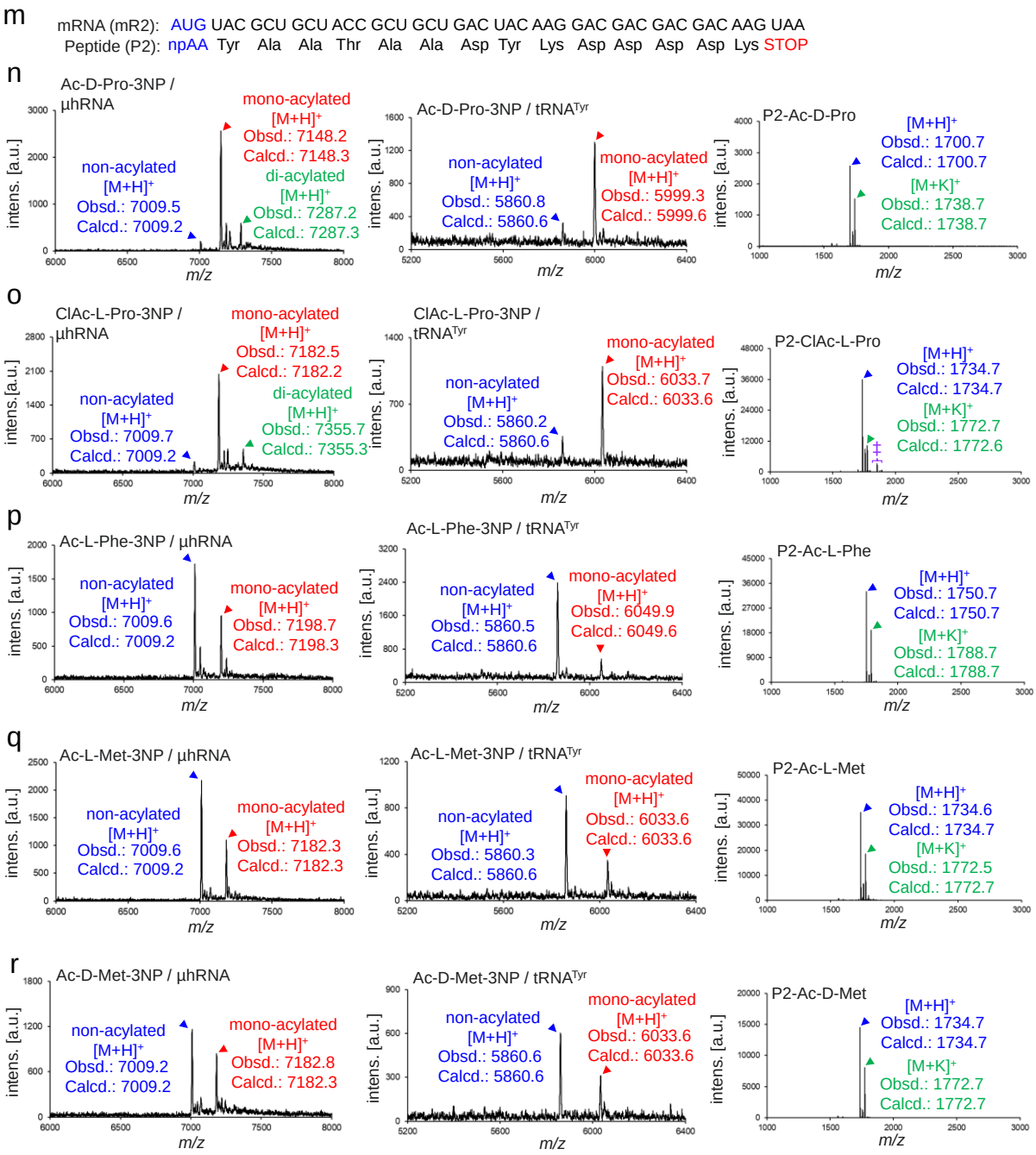
Supplementary Fig. 12 continued. Flexizyme (eFx) catalysis evaluation was conducted with (1*S*,2*S*)-2-ACHC-3NP (a), (1*S*,2*S*)-2-ACPC-3NP (b), Ac-L-Pro-3NP (c), Ac-D-Pro-3NP (d), ClAc-L-Pro-3NP (e), Cha-3NP (f), L-Lys(Tfa)-3NP (g), D-Phe(4-Cl)-3NP (h), D-Phg-3NP (i), Aib-3NP (j), Acp-3NP (k), Me-L-Leu-3NP (l), Me-L-Phe-3NP (m), Pip-3NP (n), Tic-3NP (o), Ac-L-Phe-3NP (p), Ac-L-Met-3NP (q) and Ac-D-Met-3NP (r).



Supplementary Fig. 13: MALDI-TOF MS-based evaluation of flexizyme-assisted aminoacylation utilizing 3NP class.



Supplementary Fig. 13 continued.



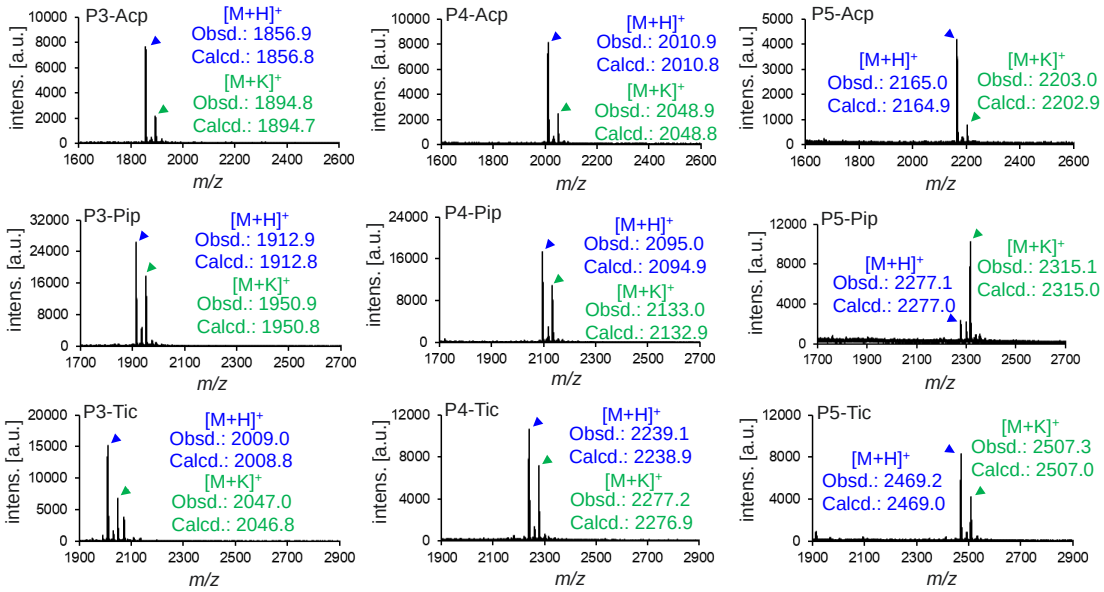
Supplementary Fig. 13 continued.

Supplementary Fig. 13 continued. μhRNA and tRNAs were acylated by flexizyme-assisted aminoacylation method using 3NP class compounds. After aminoacylation, npAA-μhRNAs were directly detected by MALDI-TOF MS. npAA-$tRNA^{Tyr}$s were treated with RNase T1, and digested 3'-terminal fragments were analyzed by MALDI-TOF MS. For the model peptide synthesis, npAA-$tRNA^{Pro1E2}_{GGU}$s were used for ribosomal elongation of npAAs. The mRNA (mR1) sequence for evaluation of npAA elongation and the corresponding peptide (P1) sequence are presented in (a). MALDI-TOF MS spectra of abovementioned experiments using (1*S*,2*S*)-2-ACPC-3NP (b), Cha-3NP (c), L-Lys(Tfa)-3NP (d), D-Phe(4-Cl)-3NP (e), D-Phg-3NP (f), Aib-3NP (g), Acp-3NP (h), Me-L-Leu-3NP (i), Me-L-Phe-3NP (j), Pip-3NP (k) and Tic-3NP (l) are presented with npAA elongation results, respectively. For the ribosomal initiation of npAAs, npAA-$tRNA^{iniP}$s acylated by the flexizyme-assisted method were used. The mRNA (mR2) sequence for evaluation of npAA initiation and the corresponding peptide (P2) sequence are presented in (m). MALDI-TOF MS spectra of the evaluation experiments utilizing Ac-D-Pro-3NP (n), ClAc-L-Pro-3NP (o), Ac-L-Phe-3NP (p), Ac-L-Met-3NP (q) and Ac-D-Met-3NP (r) are presented, respectively. "Obsd." and "Calcd." denote observed and calculated m/z values, respectively. Double dagger indicates DTT conjugated translated peptide.

a

mRNA (mR3): AUG UAC GCU GCU ACC GCU ACC GCU GCU GAC UAC AAG GAC GAC GAC GAC AAG UAA
Peptide (P3): fMet Tyr Ala Ala npAA Ala npAA Ala Ala Asp Tyr Lys Asp Asp Asp Asp Lys STOP
mRNA (mR4): AUG UAC GCU GCU ACC GCU ACC GCU ACC GCU GCU GAC UAC AAG GAC GAC GAC GAC AAG UAA
Peptide (P4): fMet Tyr Ala Ala npAA Ala npAA Ala npAA Ala Ala Asp Tyr Lys Asp Asp Asp Asp Lys STOP
mRNA (mR5): AUG UAC GCU GCU ACC GCU ACC GCU ACC GCU ACC GCU GCU GAC UAC AAG GAC GAC GAC GAC AAG UAA
Peptide (P5): fMet Tyr Ala Ala npAA Ala npAA Ala npAA Ala npAA Ala Ala Asp Tyr Lys Asp Asp Asp Asp Lys STOP

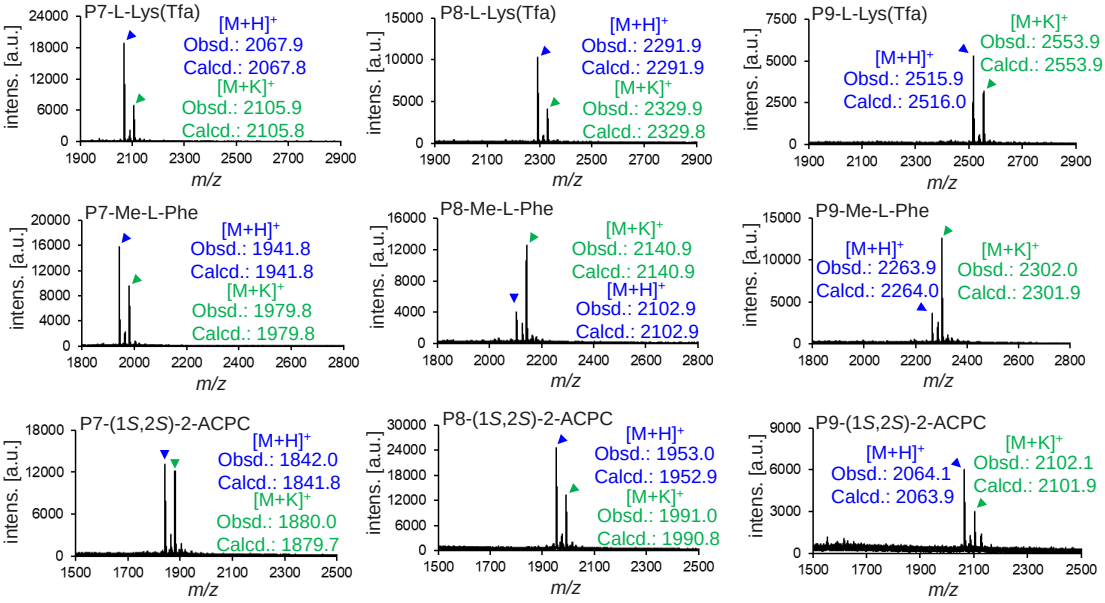
b



c

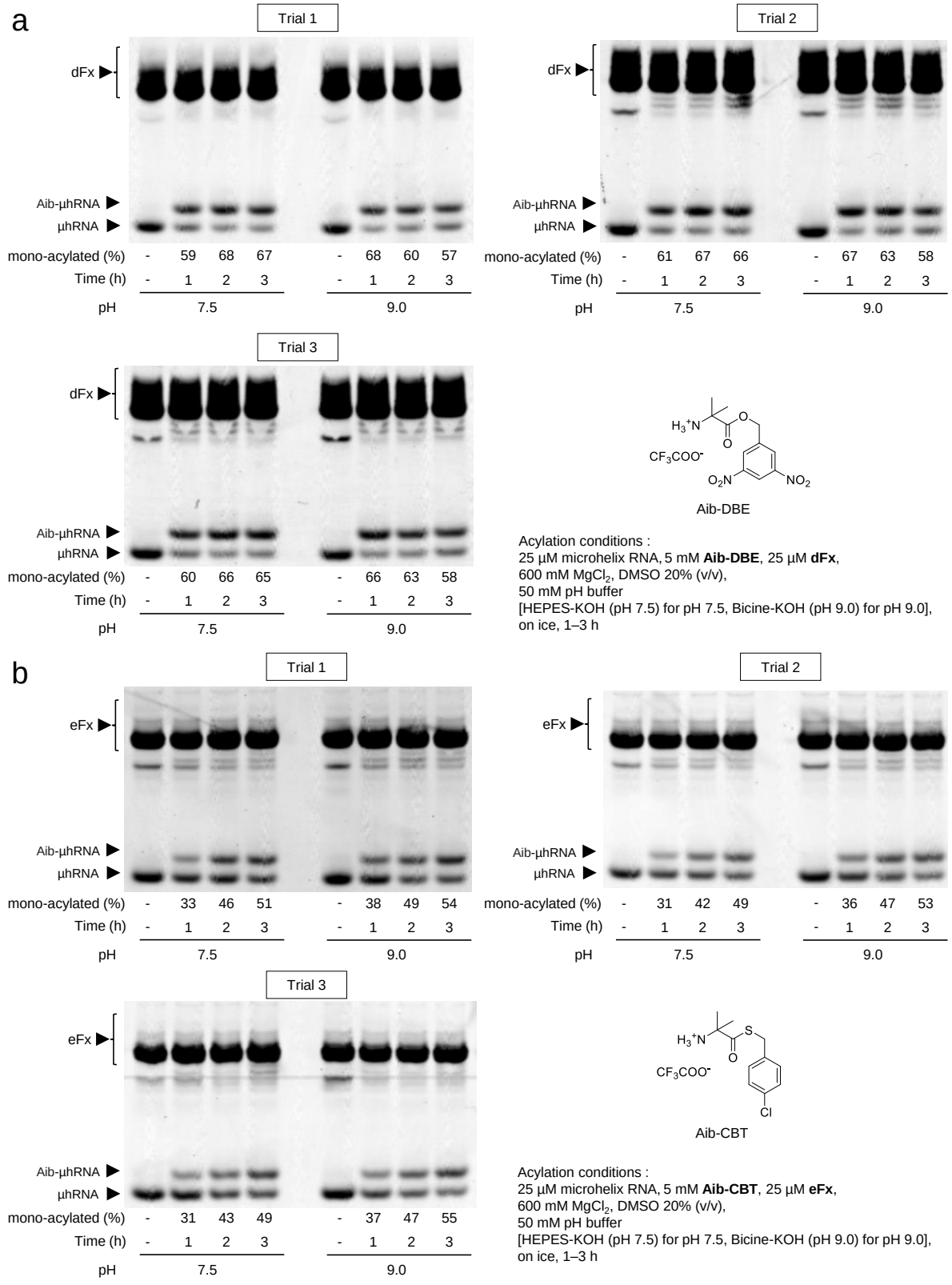
mRNA (mR7): AUG UAC GCU GCU ACC ACC GCU GCU GAC UAC AAG GAC GAC GAC GAC AAG UAA
Peptide (P7): fMet Tyr Ala Ala npAA npAA Ala Ala Asp Tyr Lys Asp Asp Asp Asp Lys STOP
mRNA (mR8): AUG UAC GCU GCU ACC ACC ACC GCU GCU GAC UAC AAG GAC GAC GAC GAC AAG UAA
Peptide (P8): fMet Tyr Ala Ala npAA npAA npAA Ala Ala Asp Tyr Lys Asp Asp Asp Asp Lys STOP
mRNA (mR9): AUG UAC GCU GCU ACC ACC ACC ACC GCU GCU GAC UAC AAG GAC GAC GAC GAC AAG UAA
Peptide (P9): fMet Tyr Ala Ala npAA npAA npAA npAA Ala Ala Asp Tyr Lys Asp Asp Asp Asp Lys STOP

d

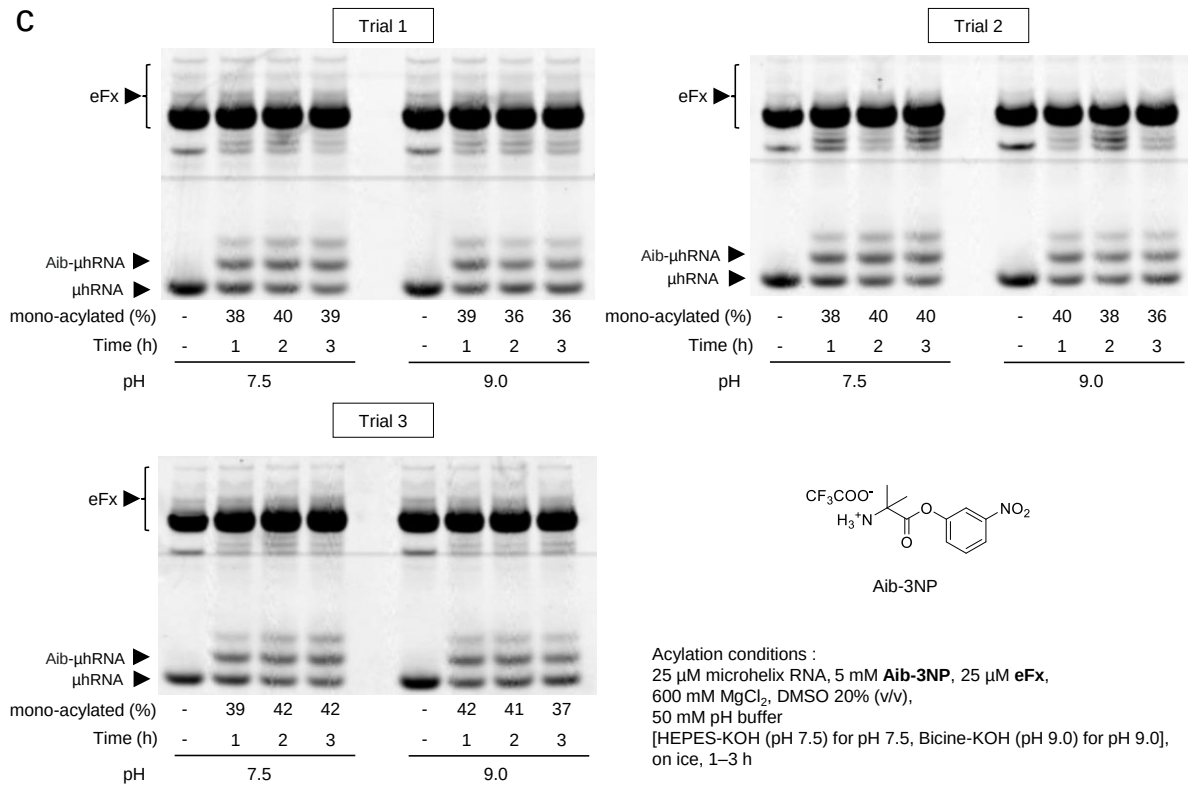


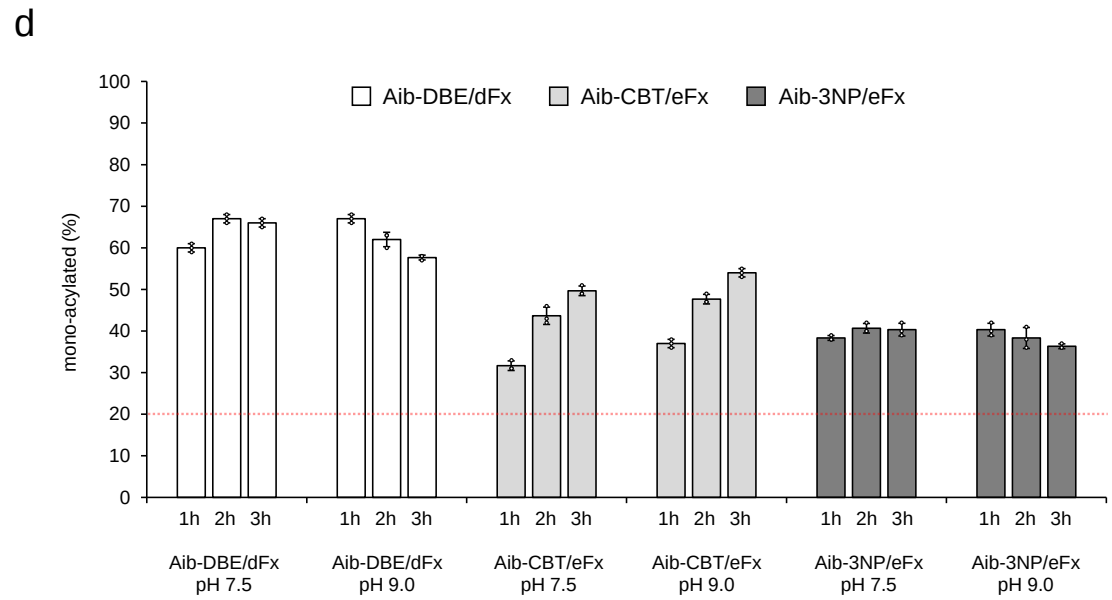
Supplementary Fig. 14: Multiple incorporation of npAAs by using npAA-tRNA acylated by flexizyme-assisted method utilizing 3NP class.

Supplementary Fig. 14 continued. a–b , non-consecutive multiple incorporation of npAAs. The mRNA (mR3-mR5) sequence for evaluation of npAA multiple incorporation and the corresponding peptide (P3-P5) sequence are presented in (a). MALDI-TOF MS spectra of ribosomal non-consecutive multiple incorporation of Acp, Pip and Tic are presented for respective npAAs in (b). **c–d ,** consecutive incorporation of npAAs. The mRNA (mR7-mR9) sequence for evaluation of npAA consecutive incorporation and the corresponding peptide (P7-P9) sequence are presented in (c). MALDI-TOF MS spectra of consecutive incorporation of L-Lys(Tfa), Me-L-Phe and (1*S*,2*S*)-2-ACPC are presented for respective npAAs in (d).



Supplementary Fig. 15: Aminoacylation comparison experiment of Aib-DBE/dFx, Aib-CBT/eFx, and Aib-3NP/eFx.





Supplementary Fig. 15 continued.

Supplementary Fig. 15 continued. a-c, μhRNA acylation reaction was conducted based on the standard flexizyme reaction in the presence of dFx or eFx. Reaction mixtures containing 25 μM dFx or eFx, 25 μM microhelix RNA, 5 mM activated amino acid, 600 mM $MgCl_2$, DMSO 20% (v/v) and 50 mM pH buffer were incubated on ice with various time points. For the activated amino acids, Aib-DBE was used in (a). Aib-CBT was tested in (b). Aib-3NP was utilized in (c). After gel pre-running at 100 V for 30 minutes, samples were loaded to the acid denaturing PAGE gel. Further gel running was conducted at 120 V for 3 hours. 50 mM sodium acetate buffer (pH 5.0) was used for gel running. μhRNA bands were stained by ethidium bromide. **d,** Graphical presentation of monoacylation yields over time, based on acid denaturing PAGE data as shown in (a), (b), and (c); n = 3, error bars; s.d.

Supplementary Table 1: Selected acylation conditions of non-enzymatic method.
Acylation reactions by non-enzymatic method were commonly conducted in 50 mM buffer containing DMSO 40% (v/v), 25 μM μhRNA or tRNA. For pH 7.0 or pH 7.5, HEPES-KOH (pH 7.0) or HEPES-KOH (pH 7.5) was used. For pH 9.5, CHES-KOH (pH 9.5) was used.

Phenol ester derivatives	Conc. of Compound (mM)	Conc. of μhRNA or tRNA (μM)	pH	Temp. (°C)	Time (h)
(1*S*,2*S*)-2-ACHC-3NP	5	25	7.5	37	2
(1*S*,2*S*)-2-ACPC-3NP	5	25	9.5	37	1
Ac-L-Pro-3NP	10	25	9.5	25	20
Ac-D-Pro-3NP	10	25	9.5	37	20
ClAc-L-Pro-3NP	10	25	9.5	37	16
Cha-3NP	2.5	25	7.0	25	3
L-Lys(Tfa)-3NP	5	25	7.0	37	3
D-Phe(4-Cl)-3NP	5	25	7.0	25	1
D-Phg-3NP	10	25	7.0	25	1
Aib-3NP	5	25	9.5	25	1
Acp-3NP	10	25	9.5	37	3
Me-L-Leu-3NP	10	25	9.5	25	12
Me-L-Phe-3NP	10	25	7.0	37	1
Pip-3NP	5	25	7.0	37	1
Tic-3NP	10	25	7.0	37	2
Ac-L-Phe-3NP	10	25	9.5	25	16
Ac-L-Met-3NP	10	25	9.5	25	16
Ac-D-Met-3NP	10	25	9.5	25	12

Supplementary Table 2: Selected acylation conditions of flexizyme-assisted method.
Acylation reactions by flexizyme-assisted method were commonly conducted in 50 mM buffer containing DMSO 20% (v/v), 600 mM $MgCl_2$, 25 µM µhRNA or tRNA and 25 µM eFx. For pH 7.0 HEPES-KOH (pH 7.0) was used. For pH 9.5, CHES-KOH (pH 9.5) was used.

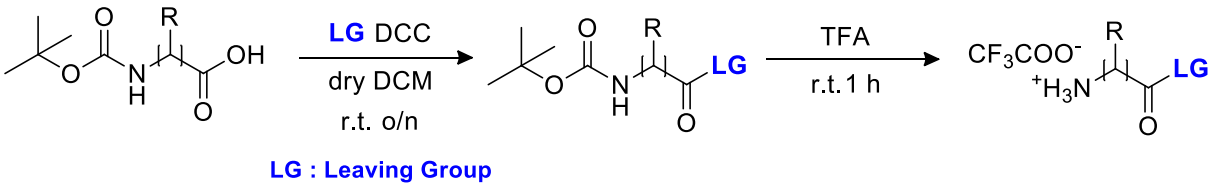
Phenol ester derivatives	**Conc. of Compound (mM)**	**Conc. of µhRNA or tRNA (µM)**	**Conc. of eFx (µM)**	**pH**	**Conc. of $MgCl_2$ (mM)**	**Temp.**	**Time (h)**
(1*S*,2*S*)-2-ACHC-3NP	5	25	25	7.0	600	on ice	2
(1*S*,2*S*)-2-ACPC-3NP	5	25	25	7.0	600	on ice	2
Ac-L-Pro-3NP	10	25	25	9.5	600	on ice	16
Ac-D-Pro-3NP	10	25	25	9.5	600	on ice	16
ClAc-L-Pro-3NP	10	25	25	9.5	600	on ice	16
Cha-3NP	5	25	25	7.0	600	on ice	2
L-Lys(Tfa)-3NP	5	25	25	7.0	600	on ice	2
D-Phe(4-Cl)-3NP	5	25	25	7.0	600	on ice	2
D-Phg-3NP	5	25	25	7.0	600	on ice	2
Aib-3NP	5	25	25	7.0	600	on ice	2
Acp-3NP	5	25	25	7.0	600	on ice	2
Me-L-Leu-3NP	5	25	25	7.0	600	on ice	2
Me-L-Phe-3NP	5	25	25	7.0	600	on ice	2
Pip-3NP	5	25	25	7.0	600	on ice	2
Tic-3NP	5	25	25	7.0	600	on ice	2
Ac-L-Phe-3NP	5	25	25	7.0	600	on ice	2
Ac-L-Met-3NP	5	25	25	7.0	600	on ice	2
Ac-D-Met-3NP	5	25	25	7.0	600	on ice	2

Materials

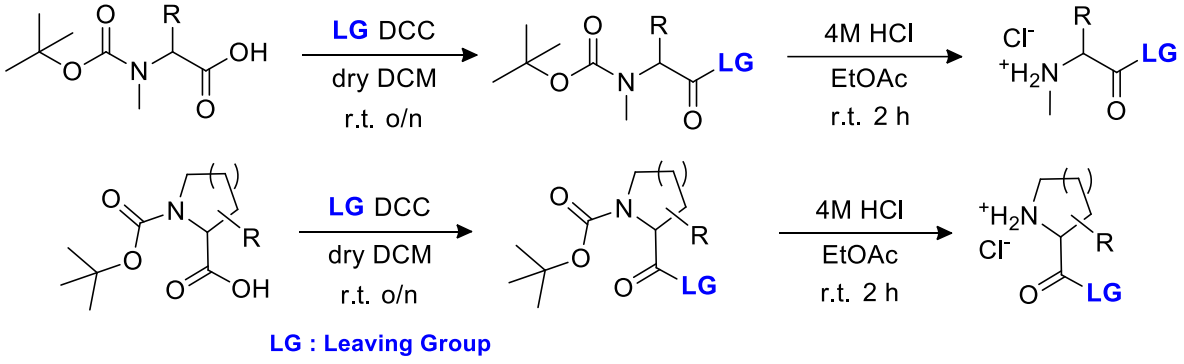
Chemical reagents were purchased from Tokyo Chemical Industry (TCI), Sigma-Aldrich, BLDpharm, Watanabe Chemical Industries, FUJIFILM Wako Pure Chemical Corporation, Kanto Chemical, and Nacalai Tesque unless otherwise described. DNA oligonucleotides were purchased from Eurofins Genomics. Microhelix RNA (μhRNA) and 2'-O-methylguanosine (Gm) containing DNA oligonucleotides were purchased from GeneDesign.

Chemical synthesis

General synthesis procedures of phenol ester derivatives are summarized in Scheme 1. Boc deprotection step was performed by using TFA or 4M HCl solution, interchangeably. For the synthesis of phenol ester derivatives for *N*-methyl amino acids and cyclic *N*-alkyl amino acids, deprotection using HCl was generally selected (Scheme 2). Further synthesis steps of other phenol ester derivatives were summarized in Scheme 3 (L-Lys(Tfa)-3NP), Scheme 4 (Ac-L-Phe-3NP), Scheme 5 (Ac-L-Met-3NP and Ac-D-Met-3NP), Scheme 6 (Ac-L-Pro-3NP and Ac-D-Pro-3NP) and Scheme 7 (ClAc-L-Pro-3NP). Synthesis steps of other activated amino acids were summarized in Scheme 8 (D-Phg-CME), Scheme 9 ((1*S*,2*S*)-2-ACHC-CBT) and Scheme 10 (Ac-L-Pro-CBT). Synthesis of DBE class compounds was generally conducted by utilizing the reported procedures.[1,2] Flash chromatography was performed by using Isolera One (Biotage). For chemical characterization, ^{1}H NMR and ^{13}C NMR spectra were obtained using JEOL ECS400 (JEOL RESONANCE). Tetramethylsilane or solvent residual was used for the reference of ^{1}H NMR and ^{13}C NMR. ESI mass spectrometry data for synthesized compounds were collected from MicrOTOF II TOF-MS (Bruker Daltonics) or compact (Bruker Daltonics). From Supplementary Fig. 16 to Supplementary Fig. 62, ^{1}H NMR and ^{13}C NMR spectra of each synthesized compounds are presented.

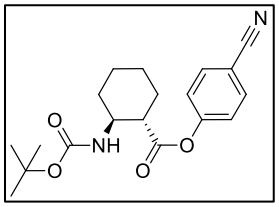


Scheme 1. General synthesis scheme of phenol ester derivatives.



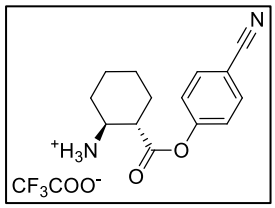
Scheme 2. Synthesis scheme of phenol ester derivatives for *N*-methyl amino acids and cyclic *N*-alkyl amino acids.

Boc-(1*S*,2*S*)-2-ACHC-4CP



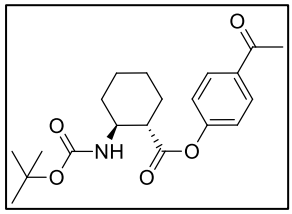
To a solution containing 103.2 mg (500 µmol) of DCC and 59.6 mg (500 µmol) of 4-hydroxybenzonitrile in dry CH_2Cl_2 2 mL, 121.6 mg (500 µmol) of Boc-(1*S*,2*S*)-2-ACHC-OH in dry CH_2Cl_2 1.5 mL was added in a portionwise manner. The mixture was stirred overnight at room temperature. After reaction, the reaction mixture was filtered and concentrated under the reduced pressure. The residual was loaded onto silica gel for purification by flash column chromatography (10:90 → 25:75 → 50:50 → 95:5 EtOAc:n-Hexane gradient elution), yielding the title compound as a white solid (102.3 mg, 59%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.69-7.64 (m, 2H), 7.26 (d, J = 8.7 Hz, 2H), 4.70 (d, J = 9.2 Hz, 1H), 3.94-3.77 (m, 1H), 2.50-2.39 (m, 1H), 2.10-2.04 (m, 2H), 1.82-1.19 (m, 15H); **^{13}C NMR** (101 MHz, $CDCl_3$) δ 171.6, 155.0, 154.1, 133.4, 122.9, 118.3, 109.5, 79.5, 51.1, 50.9, 33.2, 28.3, 24.7, 24.2; **MS** (ESI^+) *m/z*: $[M+Na]^+$ calculated for $C_{19}H_{24}N_2NaO_4^+$; 367.1628, observed; 367.1630.

(1*S*,2*S*)-2-ACHC-4CP



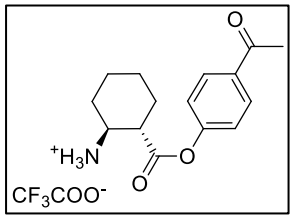
To 102.3 mg of Boc-(1*S*,2*S*)-2-ACHC-4CP, 1 mL of TFA (13.06 mmol) was added, and the mixture was left to stand for 1 hour at room temperature. After reaction, the volatile was evaporated and cold Et_2O was added in the residual. After trituration with Et_2O one time, the solid was dried under vacuum. The title compound was obtained as a white solid (100.5 mg, 94%). **^{1}H NMR** (400 MHz, DMSO-d_6) δ 8.37 (s, 3H), 7.95-7.86 (m, 2H), 7.52-7.40 (m, 2H), 3.39-3.29 (m, 1H), 2.87-2.81 (m, 1H), 2.21-1.94 (m, 2H), 1.74-1.22 (m, 6H); **^{13}C NMR** (101 MHz, DMSO-d_6) δ 171.0, 153.7, 134.0, 123.4, 118.4, 109.1, 49.6, 46.2, 29.4, 28.4, 23.8, 23.2; **MS** (ESI^+) *m/z*: $[M+H]^+$ calculated for $C_{14}H_{17}N_2O_2^+$; 245.1285, observed; 245.1281.

Boc-(1*S*,2*S*)-2-ACHC-4AP



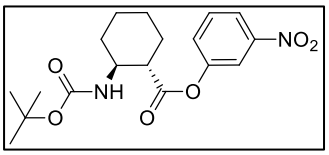
To a solution containing 103.2 mg (500 µmol) of DCC and 68.1 mg (500 µmol) of 4'-hydroxyacetophenone in dry CH_2Cl_2 2 mL, 121.6 mg (500 µmol) of Boc-(1*S*,2*S*)-2-ACHC-OH in dry CH_2Cl_2 1.5 mL was added in a portionwise manner. The mixture was stirred overnight at room temperature. After reaction, the reaction mixture was filtered and concentrated under the reduced pressure. The residual was loaded onto silica gel for purification by flash column chromatography (10:90 → 25:75 → 50:50 → 95:5 EtOAc:n-Hexane gradient elution), yielding the title compound as a white solid (56 mg, 31%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.02-7.91 (m, 2H), 7.20 (d, J = 8.7 Hz, 2H), 4.68 (d, J = 7.3 Hz, 1H), 3.88-3.71 (m, 1H), 2.59 (s, 3H), 2.53-2.43 (m, 1H), 2.12-2.07 (m, 2H), 1.81-1.16 (m, 15H); **^{13}C NMR** (101 MHz, $CDCl_3$) δ 196.9, 172.0, 155.0, 154.5, 134.7, 129.8, 121.9, 79.5, 51.3, 50.8, 33.3, 28.5, 28.4, 26.6, 24.8, 24.3; **MS** (ESI^+) *m/z*: $[M+Na]^+$ calculated for $C_{20}H_{27}NNaO_5^+$; 384.1781, observed; 384.1783.

(1*S*,2*S*)-2-ACHC-4AP



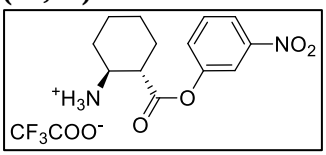
To 56 mg of Boc-(1*S*,2*S*)-2-ACHC-4AP, 1 mL of TFA (13.06 mmol) was added, and the mixture was left to stand for 1 hour at room temperature. After reaction, the volatile was evaporated and cold Et_2O was added in the residual. After trituration with Et_2O one time, the solid was dried under vacuum. The title compound was obtained as a white solid (54.1 mg, 93%). **^{1}H NMR** (400 MHz, DMSO-d_6) δ 8.30 (s, 3H), 8.08-7.98 (m, 2H), 7.43-7.35 (m, 2H), 3.40-3.33 (m, 1H), 2.86-2.76 (m, 1H), 2.58 (s, 3H), 2.23-1.99 (m, 2H), 1.73-1.23 (m, 6H); **^{13}C NMR** (101 MHz, DMSO-d_6) δ 197.0, 171.1, 153.9, 134.7, 129.8, 122.1, 49.6, 46.2, 29.3, 28.4, 26.8, 23.8, 23.1; **MS** (ESI^+) *m/z*: $[M+H]^+$ calculated for $C_{15}H_{20}NO_3^+$; 262.1438, observed; 262.1443.

Boc-(1*S*,2*S*)-2-ACHC-3NP



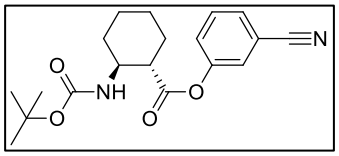
To a solution containing 103.2 mg (500 µmol) of DCC and 69.6 mg (500 µmol) of metanitrophenol in dry CH_2Cl_2 2 mL, 121.6 mg (500 µmol) of Boc-(1*S*,2*S*)-2-ACHC-OH in dry CH_2Cl_2 1.5 mL was added in a portionwise manner. The mixture was stirred overnight at room temperature. After reaction, the reaction mixture was filtered and concentrated under the reduced pressure. The residual was loaded onto silica gel for purification by flash column chromatography (10:90 → 25:75 → 50:50 → 95:5 EtOAc:n-Hexane gradient elution), yielding the title compound as a white solid (90.6 mg, 50%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.11-8.02 (m, 2H), 7.57-7.46 (m, 2H), 4.73 (d, J = 8.2 Hz, 1H), 3.90-3.77 (m, 1H), 2.53-2.47 (m, 1H), 2.14-2.07 (m, 2H), 1.90-1.20 (m, 15H); **^{13}C NMR** (101 MHz, $CDCl_3$) δ 171.9, 155.2, 151.0, 148.7, 129.9, 128.3, 120.7, 117.5, 79.9, 51.2, 50.9, 33.2, 28.4, 28.3, 24.8, 24.2; **MS** (ESI^+) *m/z*: $[M+Na]^+$ calculated for $C_{18}H_{24}N_2NaO_6^+$; 387.1527, observed; 387.1531.

(1*S*,2*S*)-2-ACHC-3NP



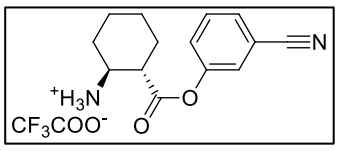
To 90.6 mg of Boc-(1*S*,2*S*)-2-ACHC-3NP, 1 mL of TFA (13.06 mmol) was added, and the mixture was left to stand for 1 hour at room temperature. After reaction, the volatile was evaporated and cold Et_2O was added in the residual. After trituration with Et_2O one time, the solid was dried under vacuum. The title compound was obtained as a white solid (84.8 mg, 90%). **^{1}H NMR** (400 MHz, DMSO-d_6) δ 8.36-8.11 (m, 5H), 7.77-7.68 (m, 2H), 3.41-3.35 (m, 1H), 2.88-2.81 (m, 1H), 2.24-1.99 (m, 2H), 1.76-1.19 (m, 6H); **^{13}C NMR** (101 MHz, DMSO-d_6) δ 171.2, 150.5, 148.3, 130.8, 128.9, 121.2, 117.6, 49.7, 46.3, 29.4, 28.4, 23.8, 23.2; **MS** (ESI^+) *m/z*: $[M+H]^+$ calculated for $C_{13}H_{17}N_2O_4^+$; 265.1183, observed; 265.1189.

Boc-(1*S*,2*S*)-2-ACHC-3CP



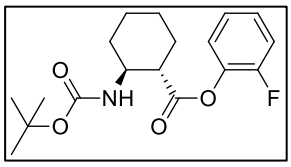
To a solution containing 103.2 mg (500 µmol) of DCC and 59.6 mg (500 µmol) of 3-cyanophenol in dry CH_2Cl_2 2 mL, 121.6 mg (500 µmol) of Boc-(1*S*,2*S*)-2-ACHC-OH in dry CH_2Cl_2 1.5 mL was added in a portionwise manner. The mixture was stirred overnight at room temperature. After reaction, the reaction mixture was filtered and concentrated under the reduced pressure. The residual was loaded onto silica gel for purification by flash column chromatography (10:90 → 25:75 → 50:50 → 95:5 EtOAc:n-Hexane gradient elution), yielding the title compound as a white solid (115.0 mg, 67%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.52-7.36 (m, 4H), 4.75 (d, J = 9.2 Hz, 1H), 3.96-3.78 (m, 1H), 2.50-2.44 (m, 1H), 2.11-2.04 (m, 2H), 1.82-1.20 (m, 15H); **^{13}C NMR** (101 MHz, $CDCl_3$) δ 171.9, 155.1, 150.8, 130.2, 129.4, 126.7, 125.6, 117.8, 113.2, 79.7, 51.2, 50.9, 33.2, 28.3, 24.7, 24.1; **MS** (ESI^+) *m/z*: $[M+Na]^+$ calculated for $C_{19}H_{24}N_2NaO_4^+$; 367.1628, observed; 367.1628.

(1*S*,2*S*)-2-ACHC-3CP



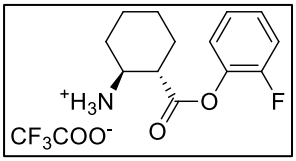
To 115.0 mg of Boc-(1*S*,2*S*)-2-ACHC-3CP, 1 mL of TFA (13.06 mmol) was added, and the mixture was left to stand for 1 hour at room temperature. After reaction, the volatile was evaporated and cold Et_2O was added in the residual. After trituration with Et_2O one time, the solid was dried under vacuum. The title compound was obtained as a white solid (102.1 mg, 85%). **^{1}H NMR** (400 MHz, DMSO-d_6) δ 8.37 (s, 3H), 7.86-7.73 (m, 2H), 7.66-7.56 (m, 2H), 3.39-3.33 (m, 1H), 2.85-2.76 (m, 1H), 2.21-2.03 (m, 2H), 1.75-1.20 (m, 6H); **^{13}C NMR** (101 MHz, DMSO-d_6) δ 171.2, 150.4, 131.0, 130.1, 127.4, 126.0, 118.0, 112.2, 49.7, 46.2, 29.4, 28.4, 23.8, 23.2; **MS** (ESI^+) *m/z*: $[M+H]^+$ calculated for $C_{14}H_{17}N_2O_2^+$; 245.1285, observed; 245.1289.

Boc-(1*S*,2*S*)-2-ACHC-2FP



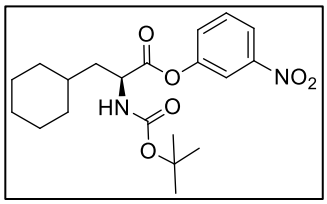
To a solution containing 103.2 mg (500 µmol) of DCC and 44.7 µL (500 µmol) of 2-fluorophenol in dry CH_2Cl_2 2 mL, 121.6 mg (500 µmol) of Boc-(1*S*,2*S*)-2-ACHC-OH in dry CH_2Cl_2 1.5 mL was added in a portionwise manner. The mixture was stirred overnight at room temperature. After reaction, the reaction mixture was filtered and concentrated under the reduced pressure. The residual was loaded onto silica gel for purification by flash column chromatography (10:90 → 25:75 → 50:50 → 95:5 EtOAc:n-Hexane gradient elution), yielding the title compound as a white solid (85.7 mg, 51 %). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.21-7.08 (m, 4H), 4.70 (s, 1H), 3.95-3.75 (m, 1H), 2.60-2.54 (m, 1H), 2.15-2.07 (m, 2H), 1.82-1.22 (m, 15H); **^{13}C NMR** (101 MHz, $CDCl_3$) δ 171.5, 155.0, 154.1 (d, J = 249.2 Hz), 138.1 (d, J = 13.4 Hz), 127.0 (d, J = 7.7 Hz), 124.3 (d, J = 3.8 Hz), 123.9, 116.5 (d, J = 18.2 Hz), 79.3, 51.2, 49.9, 33.0, 28.7, 28.3, 24.6, 24.3; **MS** (ESI^+) *m/z*: $[M+Na]^+$ calculated for $C_{18}H_{24}FNNaO_4^+$; 360.1582, observed; 360.1585.

(1*S*,2*S*)-2-ACHC-2FP



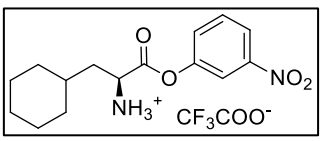
To 85.7 mg of Boc-(1*S*,2*S*)-2-ACHC-2FP, 1 mL of TFA (13.06 mmol) was added, and the mixture was left to stand for 1 hour at room temperature. After reaction, the volatile was evaporated. White precipitate was obtained from addition of Et_2O and concentration under the reduced pressure. After trituration with Et_2O one time, the solid was dried under vacuum. The title compound was obtained as a white solid (83.5 mg, 94%). **^{1}H NMR** (400 MHz, DMSO-d_6) δ 8.34 (s, 3H), 7.42-7.22 (m, 4H), 3.42-3.33 (m, 1H), 2.94-2.84 (m, 1H), 2.21-1.96 (m, 2H), 1.72-1.25 (m, 6H); **^{13}C NMR** (101 MHz, DMSO-d_6) δ 170.7, 153.4 (d, J = 247.3 Hz), 137.5 (d, J = 12.5 Hz), 127.8 (d, J = 6.7 Hz), 125.0 (d, J = 3.8 Hz), 124.5, 116.6 (d, J = 18.2 Hz), 49.4, 45.7, 29.1, 28.4, 23.8, 22.9; **MS** (ESI^+) *m/z*: $[M+H]^+$ calculated for $C_{13}H_{17}FNO_2^+$; 238.1238, observed; 238.1243.

Boc-Cha-3NP



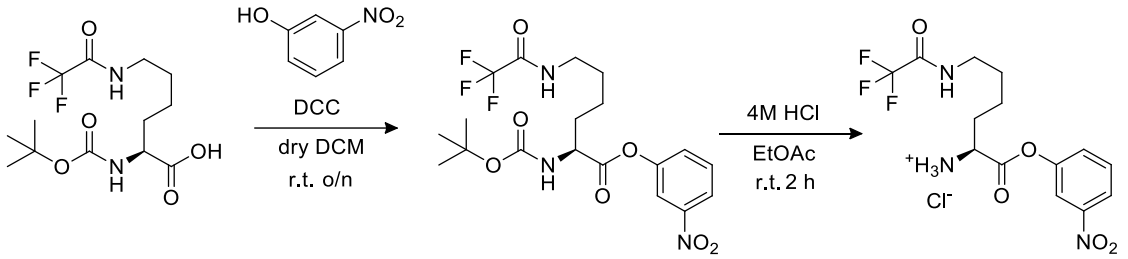
To a solution containing 206.3 mg (1 mmol) of DCC and 139.1 mg (1 mmol) of metanitrophenol in dry CH_2Cl_2 4 mL, a solution containing 271.4 mg (1 mmol) of Boc-Cha-OH in dry CH_2Cl_2 3 mL was added in a portionwise manner. The mixture was stirred overnight at room temperature. After reaction, the reaction mixture was filtered and concentrated under the reduced pressure. The residual was loaded onto silica gel for purification by flash column chromatography (10:90 → 25:75 → 50:50 → 95:5 EtOAc:n-Hexane gradient elution), yielding the title compound as a transparent oily compound (211.3 mg, 54%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.13-8.10 (m, 1H), 8.00 (t, J = 2.1 Hz, 1H), 7.57 (t, J = 8.2 Hz, 1H), 7.48 (dd, J = 8.0, 1.1 Hz, 1H), 5.30-5.10 (m, 1H), 4.57-4.43 (m, 1H), 1.93-0.84 (m, 22H); **^{13}C NMR** (101 MHz, $CDCl_3$) δ 171.7, 155.5, 150.8, 148.7, 130.0, 127.9, 120.8, 117.1, 80.2, 51.8, 39.6, 34.1, 33.5, 32.3, 28.2, 26.3, 26.1, 25.9; **MS** (ESI^+) *m/z*: $[M+Na]^+$ calculated for $C_{20}H_{28}N_2NaO_6^+$; 415.1840, observed; 415.1840.

Cha-3NP



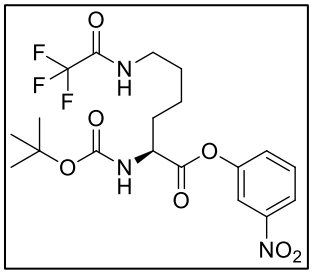
To 211.3 mg of Boc-Cha-3NP, 2 mL of TFA (26.12 mmol) was added, and the mixture was left to stand for 1 hour at room temperature. After reaction, the volatile was evaporated. White precipitate was obtained from repeating addition of Et_2O and concentration under the reduced pressure. After trituration with Et_2O one time, the solid was dried under vacuum. The title compound was obtained as a white solid (151.0 mg, 69%). **^{1}H NMR** (400 MHz, DMSO-d_6) δ 8.95 (s, 3H), 8.18-8.12 (m, 2H), 7.79-7.68 (m, 2H), 4.38-4.27 (m, 1H), 1.94-1.54 (m, 8H), 1.28-0.85 (m, 5H); **^{13}C NMR** (101 MHz, DMSO-d_6) δ 168.6, 150.1, 148.4, 131.2, 128.6, 121.6, 117.1, 50.3, 37.5, 32.9, 32.6, 32.2, 25.9,

25.7, 25.4; **MS** (ESI$^+$) *m/z*: [M+H]$^+$ calculated for $C_{15}H_{21}N_2O_4^+$; 293.1496, observed; 293.1496.



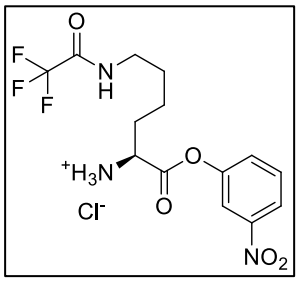
Scheme 3. Synthesis scheme of L-Lys(Tfa)-3NP.

Boc-L-Lys(Tfa)-3NP



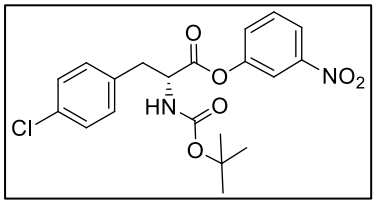
To a solution containing 103.2 mg (500 µmol) of DCC and 69.6 mg (500 µmol) of metanitrophenol in dry CH_2Cl_2 2 mL, 171.2 mg (500 µmol) of Boc-L-Lys(Tfa)-OH in dry CH_2Cl_2 5 mL was added in a portionwise manner. The mixture was stirred overnight at room temperature. After reaction, the reaction mixture was filtered and concentrated under the reduced pressure. The residual was loaded onto silica gel for purification by flash column chromatography (10:90 → 25:75 → 50:50 → 95:5 EtOAc:n-Hexane gradient elution), yielding the title compound as a slightly yellowish white solid (133.5 mg, 58%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.15-8.12 (m, 1H), 8.00 (t, J = 2.3 Hz, 1H), 7.58 (t, J = 8.0 Hz, 1H), 7.49-7.46 (m, 1H), 6.83 (s, 1H), 5.19 (d, J = 7.8 Hz, 1H), 4.52-4.42 (m, 1H), 3.48-3.36 (m, 2H), 1.90-1.32 (m, 15H); **^{13}C NMR** (101 MHz, $CDCl_3$) δ 170.9, 157.5 (q, J = 36.7 Hz), 155.6, 150.7, 148.8, 130.2, 127.9, 121.1, 117.1, 115.9 (q, J = 287.9 Hz), 80.6, 53.5, 39.5, 31.9, 28.4, 28.3, 22.6; **MS** (ESI$^+$) *m/z*: [M+Na]$^+$ calculated for $C_{19}H_{24}F_3N_3NaO_7^+$; 486.1459, observed; 486.1458.

L-Lys(Tfa)-3NP



To 133.5 mg of Boc-L-Lys(Tfa)-3NP, solution of HCl in EtOAc (4M, 4 mL) added, and the reaction mixture was kept for 2 hours at room temperature. After reaction, volatile was evaporated. The remained HCl was removed by the addition of Et_2O and concentration under reduced pressure. After trituration with Et_2O one time, the solid was dried under vacuum. The title compound was obtained as a white solid (87.6 mg, 76%). **^{1}H NMR** (400 MHz, DMSO-d_6) δ 9.54-9.44 (m, 1H), 9.00 (s, 3H), 8.22-8.13 (m, 2H), 7.84-7.72 (m, 2H), 4.33-4.18 (m, 1H), 3.26-3.12 (m, 2H), 2.09-1.85 (m, 2H), 1.62-1.47 (m, 2H), 1.36-1.06 (m, 2H); **^{13}C NMR** (101 MHz, DMSO-d_6) δ 168.0, 156.3 (q, J = 35.8 Hz), 149.9, 148.3, 131.1, 128.6, 121.6, 117.1, 116.0 (q, J = 288.2 Hz), 52.1, 38.7, 29.4, 27.7, 21.7; **MS** (ESI$^+$) *m/z*: [M+H]$^+$ calculated for $C_{14}H_{17}F_3N_3O_5^+$; 364.1115, observed; 364.1117.

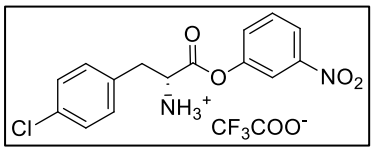
Boc-D-Phe(4-Cl)-3NP



To a solution containing 206.3 mg (1 mmol) of DCC and 139.1 mg (1 mmol) of metanitrophenol in dry CH_2Cl_2 4 mL, a solution containing 299.8 mg (1 mmol) of Boc-D-Phe(4-Cl)-OH in dry CH_2Cl_2 3 mL was added in a portionwise manner. The mixture was stirred overnight at room temperature. After reaction, the reaction mixture was filtered and concentrated under the reduced pressure. The residual was loaded onto silica

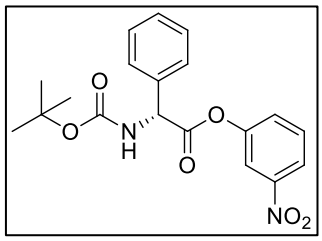
gel for purification by flash column chromatography (10:90 → 25:75 → 50:50 → 95:5 EtOAc:n-Hexane gradient elution), yielding the title compound as a white solid (123.8 mg, 29%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.11 (dd, J = 8.2, 0.9 Hz, 1H), 7.88 (t, J = 2.1 Hz, 1H), 7.56 (t, J = 8.2 Hz, 1H), 7.37-7.32 (m, 3H), 7.19 (d, J = 8.7 Hz, 2H), 5.21-5.12 (m, 1H), 4.82-4.54 (m, 1H), 3.27-3.17 (m, 2H), 1.45 (s, 9H); **^{13}C NMR** (101 MHz, $CDCl_3$) δ 170.0, 155.2, 150.5, 148.7, 134.0, 133.4, 130.7, 130.2, 129.0, 127.7, 121.1, 117.0, 80.8, 54.7, 37.5, 28.2; **MS** (ESI^+) *m/z*: $[M+Na]^+$ calculated for $C_{20}H_{21}ClN_2NaO_6^+$; 443.0980, observed; 443.0980.

D-Phe(4-Cl)-3NP



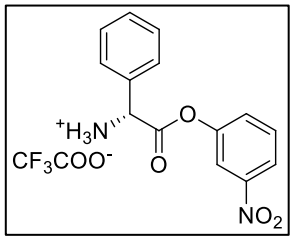
To 123.8 mg of Boc-D-Phe(4-Cl)-3NP, 1 mL of TFA (13.06 mmol) was added, and the mixture was left to stand for 1 hour at room temperature. After reaction, the volatile was evaporated and cold Et_2O was added in the residual. After trituration with Et_2O one time, the solid was dried under vacuum. The title compound was obtained as a white solid (101.7 mg, 80%). **^{1}H NMR** (400 MHz, DMSO-d_6) δ 8.99 (s, 3H), 8.16 (dd, J = 8.2, 2.3 Hz, 1H), 7.92 (t, J = 2.1 Hz, 1H), 7.75 (t, J = 8.2 Hz, 1H), 7.55 (dd, J = 8.0, 2.1 Hz, 1H), 7.46-7.38 (m, 4H), 4.67-4.57 (m, 1H), 3.38-3.28 (m, 2H); **^{13}C NMR** (101 MHz, DMSO-d_6) δ 167.6, 149.7, 148.4, 133.9, 132.4, 131.6, 131.2, 128.7, 128.4, 121.6, 116.9, 53.5, 35.3; **MS** (ESI^+) *m/z*: $[M+H]^+$ calculated for $C_{15}H_{14}ClN_2O_4^+$; 321.0637, observed; 321.0637.

Boc-D-Phg-3NP



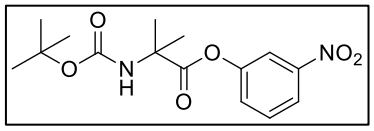
To a solution containing 103.2 mg (500 µmol) of DCC and 69.6 mg (500 µmol) of metanitrophenol in dry CH_2Cl_2 2 mL, 125.6 mg (500 µmol) of Boc-D-Phg-OH in dry CH_2Cl_2 1.5 mL was added in a portionwise manner. The mixture was stirred overnight at room temperature. After reaction, the reaction mixture was filtered and concentrated under the reduced pressure. The residual was loaded onto silica gel for purification by flash column chromatography (10:90 → 25:75 → 50:50 → 95:5 EtOAc:n-Hexane gradient elution), yielding the title compound as a white solid (130.6 mg, 70%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.13-8.07 (m, 1H), 7.94-7.86 (m, 1H), 7.54-7.30 (m, 7H), 5.58-5.28 (m, 2H), 1.47 (s, 9H); **^{13}C NMR** (101 MHz, $CDCl_3$) δ 169.4, 155.0, 150.7, 148.7, 135.2, 130.1, 129.3, 129.2, 127.8, 127.4, 121.1, 117.0, 80.7, 58.2, 28.3; **MS** (ESI^+) *m/z*: $[M+Na]^+$ calculated for $C_{19}H_{20}N_2NaO_6^+$; 395.1214, observed; 395.1214.

D-Phg-3NP



To 130.6 mg of Boc-D-Phg-3NP, 1 mL of TFA (13.06 mmol) was added, and the mixture was left to stand for 1 hour at room temperature. After reaction, the volatile was evaporated and cold Et_2O was added in the residual. After trituration with Et_2O one time, the solid was dried under vacuum. The title compound was obtained as a white solid (106 mg, 78%). **^{1}H NMR** (400 MHz, DMSO-d_6) δ 9.46 (s, 3H), 8.19-8.15 (m, 1H), 8.00 (t, J = 2.1 Hz, 1H), 7.76-7.47 (m, 7H), 5.71-5.63 (m, 1H); **^{13}C NMR** (101 MHz, DMSO-d_6) δ 167.2, 149.9, 148.5, 132.1, 131.3, 130.0, 129.3, 128.7, 128.3, 121.7, 116.8, 55.9; **MS** (ESI^+) *m/z*: $[M+H]^+$ calculated for $C_{14}H_{13}N_2O_4^+$; 273.0870, observed; 273.0867.

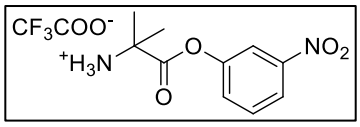
Boc-Aib-3NP



To a solution containing 206.3 mg (1 mmol) of DCC and 139.1 mg (1 mmol) of metanitrophenol in dry CH_2Cl_2 4 mL, a solution containing 203.2 mg (1 mmol) of Boc-Aib-OH in dry CH_2Cl_2 3 mL was added in a portionwise manner. The mixture was stirred overnight at room temperature. After reaction, the reaction mixture was filtered and concentrated under the reduced pressure. The residual was loaded onto silica gel for purification

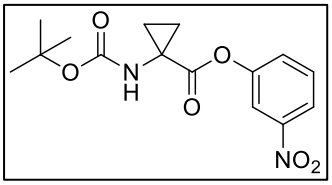
by flash column chromatography (10:90 → 25:75 → 50:50 → 95:5 EtOAc:n-Hexane gradient elution), yielding the title compound as a transparent oily compound (98.9 mg, 31%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.12-8.09 (m, 1H), 8.00 (t, J = 2.1 Hz, 1H), 7.58-7.48 (m, 2H), 5.18 (s, 1H), 1.64 (s, 6H), 1.47 (s, 9H); **^{13}C NMR** (101 MHz, $CDCl_3$) δ 173.0, 154.9, 151.4, 148.7, 130.0, 128.2, 120.8, 117.2, 80.5, 56.2, 28.3, 25.4; **MS** (ESI^+) *m/z*: $[M+Na]^+$ calculated for $C_{15}H_{20}N_2NaO_6^+$; 347.1214, observed; 347.1214.

Aib-3NP



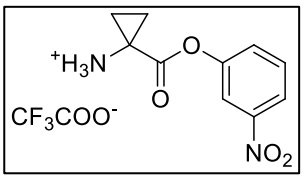
To 98.9 mg of Boc-Aib-3NP, 1 mL of TFA (13.06 mmol) was added, and the mixture was left to stand for 1 hour at room temperature. After reaction, the volatile was evaporated and cold Et_2O was added in the residual. After trituration with Et_2O one time, the solid was dried under vacuum. The title compound was obtained as a white solid (69.4 mg, 67%). **^{1}H NMR** (400 MHz, DMSO-d_6) δ 8.97 (s, 3H), 8.20-8.16 (m, 2H), 7.81-7.71 (m, 2H), 1.68 (s, 6H); **^{13}C NMR** (101 MHz, DMSO-d_6) δ 170.1, 150.1, 148.4, 131.2, 128.5, 121.7, 117.1, 56.1, 23.4; **MS** (ESI^+) *m/z*: $[M+H]^+$ calculated for $C_{10}H_{13}N_2O_4^+$; 225.0870, observed; 225.0868.

Boc-Acp-3NP



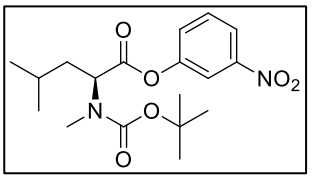
To a solution containing 103.2 mg (500 µmol) of DCC and 69.6 mg (500 µmol) of metanitrophenol in dry CH_2Cl_2 2 mL, 100.6 mg (500 µmol) of 1-(Boc-amino)cyclopropanecarboxylic acid in dry CH_2Cl_2 5 mL was added in a portionwise manner. The mixture was stirred overnight at room temperature. After reaction, the reaction mixture was filtered and concentrated under the reduced pressure. The residual was loaded onto silica gel for purification by flash column chromatography (10:90 → 25:75 → 50:50 → 95:5 EtOAc:n-Hexane gradient elution), yielding the title compound as a slightly yellowish white solid (54.6 mg, 34%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.11 (d, J = 7.8 Hz, 1H), 7.98 (t, J = 2.3 Hz, 1H), 7.56 (t, J = 8.2 Hz, 1H), 7.46 (d, J = 7.8 Hz, 1H), 5.38 (s, 1H), 1.74-1.57 (m, 4H), 1.48 (s, 9H); **^{13}C NMR** (101 MHz, $CDCl_3$) δ 171.5, 156.0, 151.1, 148.7, 130.1, 128.0, 120.8, 117.3, 80.5, 32.8, 28.3, 18.8; **MS** (ESI^+) *m/z*: $[M+Na]^+$ calculated for $C_{15}H_{18}N_2NaO_6^+$; 345.1057, observed; 345.1060.

Acp-3NP



To 54.6 mg of Boc-Acp-3NP, 1 mL of TFA (13.06 mmol) was added, and the mixture was left to stand for 1 hour at room temperature. After reaction, the volatile was evaporated and cold Et_2O was added in the residual. After trituration with Et_2O one time, the solid was dried under vacuum. The title compound was obtained as a white solid (43.7 mg, 77 %). **^{1}H NMR** (400 MHz, DMSO-d_6) δ 9.13 (s, 3H), 8.19-8.13 (m, 2H), 7.78-7.68 (m, 2H), 1.78-1.71 (m, 2H), 1.56-1.51 (m, 2H); **^{13}C NMR** (101 MHz, DMSO-d_6) δ 168.4, 150.0, 148.4, 131.1, 128.6, 121.5, 117.1, 34.0, 14.6; **MS** (ESI^+) *m/z*: $[M+H]^+$ calculated for $C_{10}H_{11}N_2O_4^+$; 223.0713, observed; 223.0715.

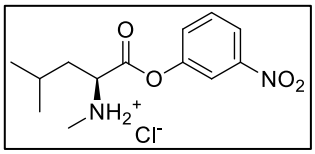
Boc-Me-L-Leu-3NP



To a solution containing 206.3 mg (1 mmol) of DCC and 139.1 mg (1 mmol) of metanitrophenol in dry CH_2Cl_2 4 mL, a solution containing 245.3 mg (1 mmol) of Boc-Me-L-Leu-OH in dry CH_2Cl_2 3 mL was added in a portionwise manner. The mixture was stirred overnight at room temperature. After reaction, the reaction mixture was filtered and concentrated under the reduced pressure. The residual was loaded onto silica gel for purification by flash column chromatography (10:90 → 25:75 → 50:50 → 95:5 EtOAc:n-Hexane gradient elution), yielding the title compound as a transparent oily compound (174.2 mg, 48%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.12-7.95 (m, 2H), 7.61-7.46 (m, 2H), 5.01-4.80 (m, 1H), 2.92 (s, 3H), 1.97-1.63 (m, 3H), 1.50 (s, 9H), 1.07-1.00

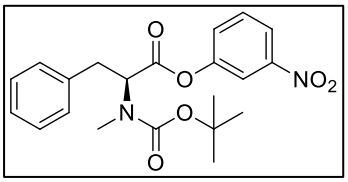
(m, 6H); **^{13}C NMR** (101 MHz, $CDCl_3$) δ 170.7, 156.1, 151.0, 148.7, 130.0, 128.0, 120.8, 117.2, 80.4, 56.7, 37.5, 31.3, 28.3, 24.9, 23.1, 21.3; **MS** (ESI^+) *m/z*: $[M+Na]^+$ calculated for $C_{18}H_{26}N_2NaO_6^+$; 389.1683, observed; 389.1685.

Me-L-Leu-3NP



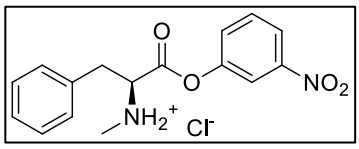
To 174.2 mg of Boc-Me-L-Leu-3NP, solution of HCl in EtOAc (4M, 8 mL) added, and the reaction mixture was kept for 2 hours at room temperature. After reaction, volatile was evaporated. The remained HCl was removed by the addition of Et_2O and concentration under reduced pressure. After trituration with Et_2O one time, the solid was dried under vacuum. The title compound was obtained as a white solid (119.7 mg, 83%). **^{1}H NMR** (400 MHz, DMSO-d_6) δ 10.16 (s, 2H), 8.24-8.17 (m, 2H), 7.81-7.75 (m, 2H), 4.31-4.22 (m, 1H), 2.67 (s, 3H), 1.99-1.81 (m, 3H), 1.01-0.95 (m, 6H); **^{13}C NMR** (101 MHz, DMSO-d_6) δ 167.4, 149.7, 148.3, 131.1, 128.7, 121.7, 117.2, 58.2, 37.1, 30.9, 24.3, 22.9, 21.4; **MS** (ESI^+) *m/z*: $[M+H]^+$ calculated for $C_{13}H_{19}N_2O_4^+$; 267.1339, observed; 267.1345.

Boc-Me-L-Phe-3NP



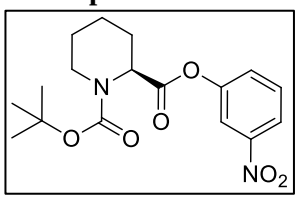
To a solution containing 103.2 mg (500 µmol) of DCC and 69.6 mg (500 µmol) of metanitrophenol in dry CH_2Cl_2 2 mL, a solution containing 139.7 mg (500 µmol) of Boc-Me-L-Phe-OH in dry CH_2Cl_2 1.5 mL was added in a portionwise manner. The mixture was stirred overnight at room temperature. After reaction, the reaction mixture was filtered and concentrated under the reduced pressure. The residual was loaded onto silica gel for purification by flash column chromatography (10:90 → 25:75 → 50:50 → 95:5 EtOAc:n-Hexane gradient elution), yielding the title compound as a transparent oily compound (67 mg, 33%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.12-7.96 (m, 2H), 7.55-7.44 (m, 2H), 7.33-7.26 (m, 5H), 4.78-4.75 (m, 1H), 3.40-3.18 (m, 2H), 2.85-2.77 (m, 3H), 1.45-1.32 (m, 9H); **^{13}C NMR** (101 MHz, $CDCl_3$) δ 169.7, 155.7, 151.1, 148.8, 137.0, 130.0, 129.1, 128.6, 128.2, 126.8, 120.9, 117.3, 80.6, 61.5, 35.2, 34.0, 28.3; **MS** (ESI^+) *m/z*: $[M+Na]^+$ calculated for $C_{21}H_{24}N_2NaO_6^+$; 423.1527, observed; 423.1527.

Me-L-Phe-3NP



To 67 mg of Boc-Me-L-Phe-3NP, solution of HCl in EtOAc (4M, 4 mL) added, and the reaction mixture was kept for 2 hours at room temperature. After reaction, volatile was evaporated. The remained HCl was removed by the addition of Et_2O and concentration under reduced pressure. After trituration with Et_2O one time, the solid was dried under vacuum. The title compound was obtained as a white solid (52 mg, 92%). **^{1}H NMR** (400 MHz, DMSO-d_6) δ 10.23 (s, 2H), 8.15-8.13 (m, 1H), 7.80 (t, J = 2.1 Hz, 1H), 7.72 (t, J = 8.2 Hz, 1H), 7.46-7.29 (m, 6H), 4.62-4.55 (m, 1H), 3.63-3.56 (m, 1H), 3.28-3.19 (m, 1H), 2.74 (s, 3H); **^{13}C NMR** (101 MHz, DMSO-d_6) δ 166.9, 149.4, 148.2, 134.5, 131.1, 129.7, 128.7, 128.4, 127.6, 121.7, 116.8, 60.8, 34.5, 31.3; **MS** (ESI^+) *m/z*: $[M+H]^+$ calculated for $C_{16}H_{17}N_2O_4^+$; 301.1183, observed; 301.1183.

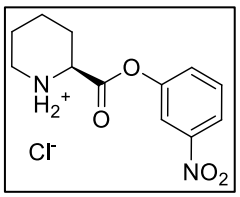
Boc-Pip-3NP



To a solution containing 103.2 mg (500 µmol) of DCC and 69.6 mg (500 µmol) of metanitrophenol in dry CH_2Cl_2 2 mL, 114.6 mg (500 µmol) of Boc-Pip-OH in dry CH_2Cl_2 1.5 mL was added in a portionwise manner. The mixture was stirred overnight at room temperature. After reaction, the reaction mixture was filtered and concentrated under the reduced pressure. The residual was loaded onto silica gel for purification by flash column chromatography (10:90 → 25:75 → 50:50 → 95:5 EtOAc:n-Hexane gradient elution), yielding the title compound as a transparent oily compound (39.0 mg, 22%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.13 (d, J = 7.3 Hz, 1H), 7.99 (s, 1H),

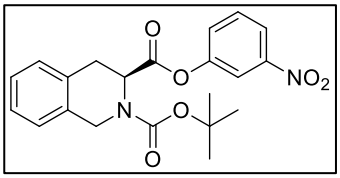
7.60-7.40 (m, 2H), 5.14-4.79 (m, 1H), 4.13-3.95 (m, 1H), 3.14-2.99 (m, 1H), 2.38-2.28 (m, 1H), 1.89-1.34 (m, 14H); **^{13}C NMR** (101 MHz, $CDCl_3$) δ 170.5, 156.1, 151.1, 148.8, 130.1, 128.1, 120.9, 117.3, 80.5, 54.1, 42.3, 28.4, 26.8, 24.7, 21.1; **MS** (ESI^+) *m/z*: $[M+Na]^+$ calculated for $C_{17}H_{22}N_2NaO_6^+$; 373.1370, observed; 373.1369.

Pip-3NP



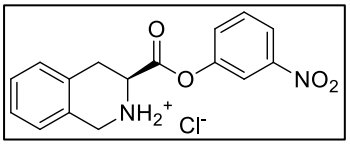
To 39.0 mg of Boc-Pip-3NP, solution of HCl in EtOAc (4M, 4 mL) added, and the reaction mixture was kept for 2 hours at room temperature. After reaction, volatile was evaporated. The remained HCl was removed by the addition of Et_2O and concentration under reduced pressure. After trituration with Et_2O one time, the solid was dried under vacuum. The title compound was obtained as a white solid (31.4 mg, 99 %). **^{1}H NMR** (400 MHz, DMSO-d_6) δ 10.11 (s, 1H), 9.78 (s, 1H), 8.24-8.15 (m, 2H), 7.83-7.73 (m, 2H), 4.38 (s, 1H), 3.31 (d, J = 12.8 Hz, 1H), 2.98 (s, 1H), 2.27-2.22 (m, 1H), 1.99-1.57 (m, 5H); **^{13}C NMR** (101 MHz, DMSO-d_6) δ 167.2, 149.9, 148.3, 131.2, 128.6, 121.6, 117.1, 55.7, 43.4, 25.3, 21.1, 21.1; **MS** (ESI^+) *m/z*: $[M+H]^+$ calculated for $C_{12}H_{15}N_2O_4^+$; 251.1026, observed; 251.1029.

Boc-Tic-3NP



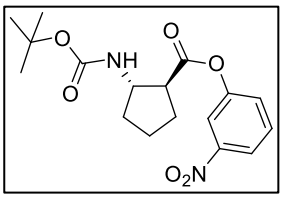
To a solution containing 206.3 mg (1 mmol) of DCC and 139.1 mg (1 mmol) of metanitrophenol in dry CH_2Cl_2 4 mL, a solution containing 277.3 mg (1 mmol) of Boc-Tic-OH in dry CH_2Cl_2 3 mL was added in a portionwise manner. The mixture was stirred overnight at room temperature. After reaction, the reaction mixture was filtered and concentrated under the reduced pressure. The residual was loaded onto silica gel for purification by flash column chromatography (10:90 → 25:75 → 50:50 → 95:5 EtOAc:n-Hexane gradient elution), yielding the title compound as a transparent oily compound (194.1 mg, 49%). **^{1}H NMR** (400 MHz, $CDCl_3$) (mixture of rotamers) δ 8.06-8.01 (m, 1H), 7.71-7.68 (m, 1H), 7.50-7.43 (m, 1H), 7.29-7.14 (m, 5H), 5.33-5.06 (m, 1H), 4.76-4.58 (m, 2H), 3.42-3.28 (m, 2H), 1.60-1.48 (m, 9H); **^{13}C NMR** (101 MHz, $CDCl_3$) (mixture of rotamers) δ 170.1, 169.8, 155.4, 154.6, 150.7, 150.6, 149.0, 148.6, 133.9, 133.1, 131.8, 131.6, 130.0, 129.9, 128.1, 127.8, 127.6, 127.5, 127.4, 127.3, 127.2, 127.1, 126.5, 126.3, 120.8, 120.7, 117.0, 116.8, 81.2, 81.2, 54.8, 53.4, 44.9, 44.3, 32.0, 31.5, 28.3; **MS** (ESI^+) *m/z*: $[M+Na]^+$ calculated for $C_{21}H_{22}N_2NaO_6^+$; 421.1370, observed; 421.1369.

Tic-3NP



To 194.1 mg of Boc-Tic-3NP, solution of HCl in EtOAc (4M, 8 mL) added, and the reaction mixture was kept for 2 hours at room temperature. After reaction, volatile was evaporated. The remained HCl was removed by the addition of Et_2O and concentration under reduced pressure. After trituration with Et_2O one time, the solid was dried under vacuum. The title compound was obtained as a white solid (119.1 mg, 73%). **^{1}H NMR** (400 MHz, DMSO-d_6) δ 10.80 (s, 2H), 8.24-8.15 (m, 2H), 7.84-7.76 (m, 2H), 7.31-7.24 (m, 4H), 4.91-4.78 (m, 1H), 4.46-4.35 (m, 2H), 3.55-3.42 (m, 2H); **^{13}C NMR** (101 MHz, DMSO-d_6) δ 166.8, 149.9, 148.3, 131.2, 130.4, 128.7, 128.6, 128.4, 127.6, 126.9, 126.6, 121.7, 117.1, 53.2, 43.8, 27.8; **MS** (ESI^+) *m/z*: $[M+H]^+$ calculated for $C_{16}H_{15}N_2O_4^+$; 299.1026, observed; 299.1031. .

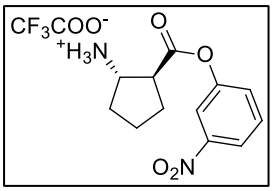
Boc-(1*S*,2*S*)-2-ACPC-3NP



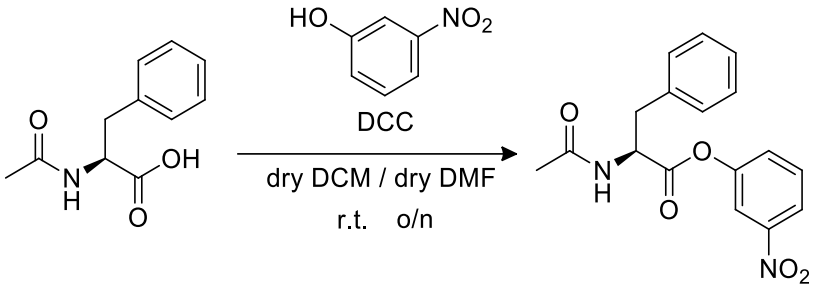
To a solution containing 103.2 mg (500 µmol) of DCC and 69.6 mg (500 µmol) of metanitrophenol in dry CH_2Cl_2 2 mL, a mixture containing 114.6 mg (500 µmol) of Boc-(1*S*,2*S*)-2-ACPC-OH in dry CH_2Cl_2 1.5 mL was added in a portionwise manner. The mixture was stirred overnight at room temperature. After reaction, the reaction mixture was filtered and concentrated under the reduced pressure. The

residual was loaded onto silica gel for purification by flash column chromatography (CH_2Cl_2 100%, isocratic elution), yielding the title compound as a slightly yellowish white solid (76 mg, 43%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.11-8.08 (m, 2H), 7.55-7.46 (m, 2H), 4.82 (d, J = 5.0 Hz, 1H), 4.35-4.27 (m, 1H), 2.84-2.78 (m, 1H), 2.27-1.40 (m, 15H); **^{13}C NMR** (101 MHz, $CDCl_3$) δ 173.1, 155.6, 151.2, 148.7, 129.9, 128.4, 120.7, 117.5, 79.9, 56.6, 51.5, 32.9, 28.6, 28.3, 22.7; **MS** (ESI^+) *m/z*: $[M+Na]^+$ calculated for $C_{17}H_{22}N_2NaO_6^+$; 373.1370, observed; 373.1375.

(1*S*,2*S*)-2-ACPC-3NP

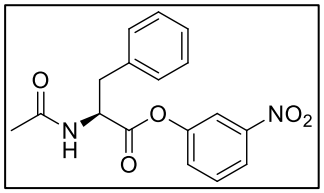


To 76 mg of Boc-(1*S*,2*S*)-2-ACPC-3NP, 2 mL of TFA (26.12 mmol) was added, and the mixture was left to stand for 1 hour at room temperature. After reaction, the volatile was evaporated. White precipitate was obtained from addition of Et_2O and concentration under the reduced pressure. After trituration with Et_2O one time, the solid was dried under vacuum. The title compound was obtained as a white solid (61 mg, 77%). **^{1}H NMR** (400 MHz, DMSO-d_6) δ 8.44 (s, 3H), 8.19-8.14 (m, 2H), 7.83-7.70 (m, 2H), 3.96-3.87 (m, 1H), 3.26-3.20 (m, 1H), 2.30-1.68 (m, 6H); **^{13}C NMR** (101 MHz, DMSO-d_6) δ 171.7, 150.7, 148.3, 130.8, 128.9, 121.1, 117.5, 53.4, 48.0, 31.1, 29.3, 23.4; **MS** (ESI^+) *m/z*: $[M+H]^+$ calculated for $C_{12}H_{15}N_2O_4^+$; 251.1026, observed; 251.1025.

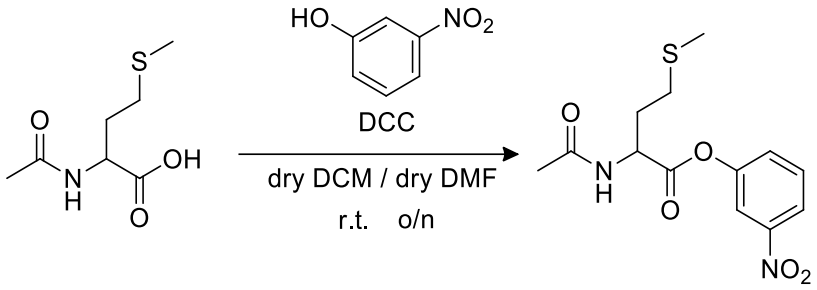


Scheme 4. Synthesis scheme of Ac-L-Phe-3NP.

Ac-L-Phe-3NP

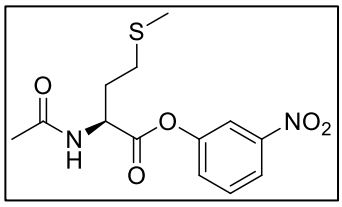


To a solution containing 206.3 mg (1 mmol) of DCC and 139.1 mg (1 mmol) of metanitrophenol in dry CH_2Cl_2 4 mL, a solution containing 207.2 mg (1 mmol) of *N*-acetyl-L-phenylalanine in dry DMF 500 µL was added in a portionwise manner. The mixture was stirred overnight at room temperature. After reaction, the reaction mixture was filtered in a bed of celite. Filtered precipitate was washed with CH_2Cl_2 10 mL to recover the product. After concentration under reduced pressure, the residual was loaded onto silica gel for purification by flash column chromatography (10:90 → 30:70 → 50:50 → 99:1 EtOAc:n-Hexane gradient elution), yielding the title compound as a slightly yellowish white solid (49.4 mg, 15%). **^{1}H NMR** (400 MHz, DMSO-d_6) δ 8.63 (d, J = 6.4 Hz, 1H), 8.13-8.09 (m, 1H), 7.76-7.67 (m, 2H), 7.44-7.24 (m, 6H), 4.69-4.62 (m, 1H), 3.22-3.09 (m, 2H), 1.88 (s, 3H); **^{13}C NMR** (101 MHz, DMSO-d_6) δ 170.4, 169.9, 150.5, 148.2, 136.9, 130.9, 129.3, 128.5, 128.4, 126.8, 121.0, 116.8, 54.3, 36.3, 22.1; **MS** (ESI^+) *m/z*: $[M+Na]^+$ calculated for $C_{17}H_{16}N_2NaO_5^+$, 351.0951, observed; 351.0948.



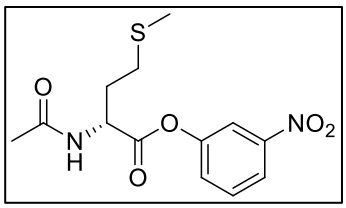
Scheme 5. Common synthesis scheme of Ac-L-Met-3NP and Ac-D-Met-3NP.

Ac-L-Met-3NP

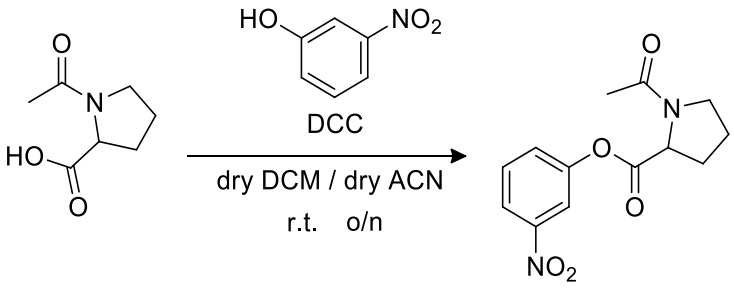


To a solution containing 206.3 mg (1 mmol) of DCC and 139.1 mg (1 mmol) of metanitrophenol in dry CH_2Cl_2 4 mL, a solution containing 191.3 mg (1 mmol) of Ac-L-Met-OH in dry DMF 500 µL was added in a portionwise manner. The mixture was stirred overnight at room temperature. After reaction, the reaction mixture was filtered and concentrated. The residual was loaded onto silica gel for purification by flash column chromatography (10:90 → 25:75 → 50:50 → 95:5 EtOAc:n-Hexane gradient elution), yielding the title compound as a white solid (90 mg, 29%). **^{1}H NMR** (400 MHz, DMSO-d_6) δ 8.56 (d, J = 6.9 Hz, 1H), 8.14-8.11 (m, 1H), 8.00 (t, J = 2.3 Hz, 1H), 7.72 (t, J = 8.2 Hz, 1H), 7.61-7.58 (m, 1H), 4.58-4.52 (m, 1H), 2.71-2.53 (m, 2H), 2.24-1.99 (m, 5H), 1.91 (s, 3H); **^{13}C NMR** (101 MHz, DMSO-d_6) δ 170.7, 170.3, 150.7, 148.4, 131.0, 128.8, 121.1, 117.1, 51.6, 30.0, 29.6, 22.2, 14.6; **MS** (ESI^+) *m/z*: $[M+Na]^+$ calculated for $C_{13}H_{16}N_2NaO_5S^+$, 335.0672, observed; 335.0676.

Ac-D-Met-3NP

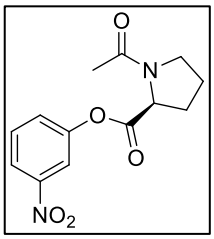


To a solution containing 206.3 mg (1 mmol) of DCC and 139.1 mg (1 mmol) of metanitrophenol in dry CH_2Cl_2 4 mL, a solution containing 191.3 mg (1 mmol) of Ac-D-Met-OH in dry DMF 500 µL was added in a portionwise manner. The mixture was stirred overnight at room temperature. After reaction, the reaction mixture was filtered and concentrated. The residual was loaded onto silica gel for purification by flash column chromatography (10:90 → 25:75 → 50:50 → 95:5 EtOAc:n-Hexane gradient elution), yielding the title compound as a white solid (65 mg, 21%). **^{1}H NMR** (400 MHz, DMSO-d_6) δ 8.54 (d, J = 6.9 Hz, 1H), 8.16-8.13 (m, 1H), 8.01 (t, J = 2.3 Hz, 1H), 7.73 (t, J = 8.2 Hz, 1H), 7.62-7.59 (m, 1H), 4.57-4.52 (m, 1H), 2.72-2.54 (m, 2H), 2.22-2.01 (m, 5H), 1.91 (s, 3H); **^{13}C NMR** (101 MHz, DMSO-d_6) δ 170.6, 170.0, 150.6, 148.3, 130.9, 128.7, 121.0, 117.0, 51.5, 29.9, 29.5, 22.1, 14.5; **MS** (ESI^+) *m/z*: $[M+Na]^+$ calculated for $C_{13}H_{16}N_2NaO_5S^+$, 335.0672, observed; 335.0671.



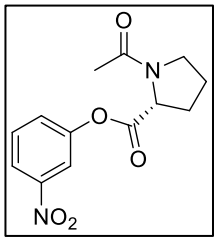
Scheme 6. Common synthesis scheme of Ac-L-Pro-3NP and Ac-D-Pro-3NP.

Ac-L-Pro-3NP

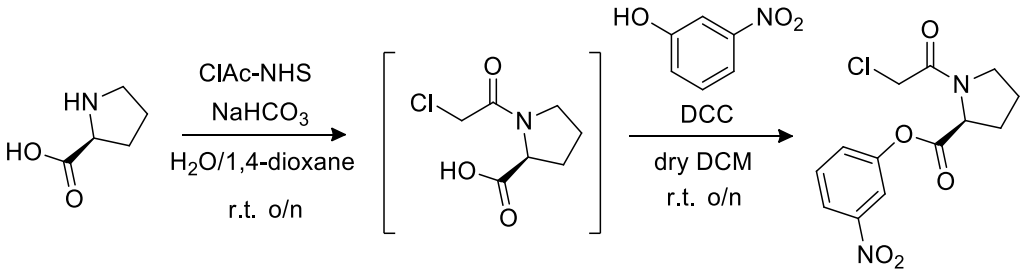


To a solution containing 206.3 mg (1 mmol) of DCC and 139.1 mg (1 mmol) of metanitrophenol in dry CH_2Cl_2 4 mL, a solution containing 157.2 mg (1 mmol) of *N*-acetyl-L-proline in dry ACN 3 mL was added in a portionwise manner. The mixture was stirred overnight at room temperature. After reaction, the reaction mixture was filtered and concentrated. The residual was loaded onto silica gel for purification by flash column chromatography (10:90 → 25:75 → 50:50 → 95:5 EtOAc:n-Hexane gradient elution), yielding the title compound as a transparent oily compound (134.8 mg, 48%). **^{1}H NMR** (400 MHz, DMSO-d_6) δ 8.16-8.12 (m, 1H), 7.97 (t, J = 2.1 Hz, 1H), 7.76-7.69 (m, 1H), 7.60-7.57 (m, 1H), 4.54-4.43 (m, 1H), 3.65-3.40 (m, 2H), 2.38-1.95 (m, 7H); **^{13}C NMR** (101 MHz, DMSO-d_6) δ 170.7, 168.9, 150.7, 148.3, 130.9, 128.6, 120.9, 116.9, 58.4, 47.3, 28.9, 24.8, 21.8; **MS** (ESI$^+$) *m/z*: $[M+Na]^+$ calculated for $C_{13}H_{14}N_2NaO_5^+$; 301.0795, observed; 301.0795.

Ac-D-Pro-3NP

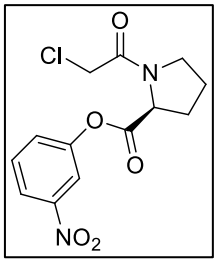


To a solution containing 206.3 mg (1 mmol) of DCC and 139.1 mg (1 mmol) of metanitrophenol in dry CH_2Cl_2 4 mL, a solution containing 157.2 mg (1 mmol) of *N*-acetyl-D-proline in dry ACN 3 mL was added in a portionwise manner. The mixture was stirred overnight at room temperature. After reaction, the reaction mixture was filtered and concentrated. The residual was loaded onto silica gel for purification by flash column chromatography (10:90 → 25:75 → 50:50 → 95:5 EtOAc:n-Hexane gradient elution), yielding the title compound as a transparent oily compound (100.4 mg, 36%). **^{1}H NMR** (400 MHz, DMSO-d_6) δ 8.16-8.11 (m, 1H), 7.97 (t, J = 2.3 Hz, 1H), 7.74-7.69 (m, 1H), 7.60-7.57 (m, 1H), 4.49-4.42 (m, 1H), 3.64-3.54 (m, 2H), 2.34-1.98 (m, 7H); **^{13}C NMR** (101 MHz, DMSO-d_6) δ 170.8, 169.1, 150.7, 148.3, 131.0, 128.7, 121.1, 117.0, 58.5, 47.5, 28.9, 24.8, 21.9; **MS** (ESI$^+$) *m/z*: $[M+Na]^+$ calculated for $C_{13}H_{14}N_2NaO_5^+$; 301.0795, observed; 301.0800.



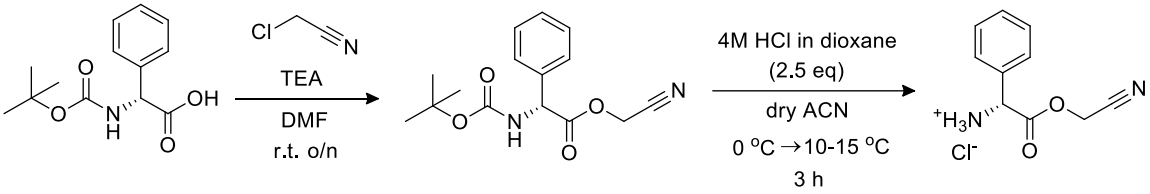
Scheme 7. Synthesis scheme of ClAc-L-Pro-3NP.

ClAc-L-Pro-3NP



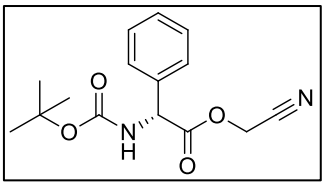
Introduction of *N*-chloroacetyl moiety to amino acids was performed as previously described.[3] A mixture containing L-proline (115.1 mg, 1 mmol) , *N*-(Chloroacetoxy)succinimide (243.3 mg, 1.27 mmol) and $NaHCO_3$ (263.0 mg, 3.13 mmol) in 16 mL of 50% (v/v) 1,4-dioxane (aq.) was stirred overnight at room temperature. After reaction, the reaction mixture was acidified by addition of $KHSO_4$ (1.025 g, 7.53 mmol). After the extraction with 20 mL of EtOAc four times, the combined organic layer was washed with 10 mL of brine three times. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The resulting compound (ClAc-L-Pro-OH) was dissolved in dry DCM and added to a solution containing 206.3 mg (1 mmol) of DCC and 139.1 mg (1 mmol) of metanitrophenol in dry CH_2Cl_2 4 mL in a portionwise manner. The mixture was stirred overnight at room temperature. After reaction, the reaction mixture was filtered and concentrated. The residual was loaded onto silica gel for purification by flash column chromatography (10:90 → 25:75 → 50:50 → 95:5 EtOAc:n-Hexane gradient elution), yielding the title compound as a transparent oily compound (51.6 mg, 17%). **^{1}H NMR** (400 MHz, DMSO-

d_6) δ 8.19-8.16 (m, 1H), 8.02 (t, J = 2.3 Hz, 1H), 7.76 (t, J = 8.2 Hz, 1H), 7.63-7.61 (m, 1H), 4.60-4.34 (m, 3H), 3.73-3.45 (m, 2H), 2.42-1.85 (m, 4H); **^{13}C NMR** (101 MHz, DMSO-d_6) δ 170.1, 165.0, 150.5, 148.3, 131.0, 128.6, 121.1, 116.9, 59.1, 46.6, 42.4, 28.5, 24.8; **MS** (ESI^+) *m/z*: $[M+Na]^+$ calculated for $C_{13}H_{13}ClN_2NaO_5^+$; 335.0405, observed; 335.0404.



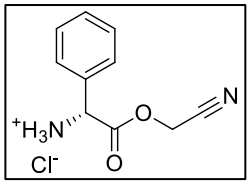
Scheme 8. Synthesis scheme of D-Phg-CME.

Boc-D-Phg-CME

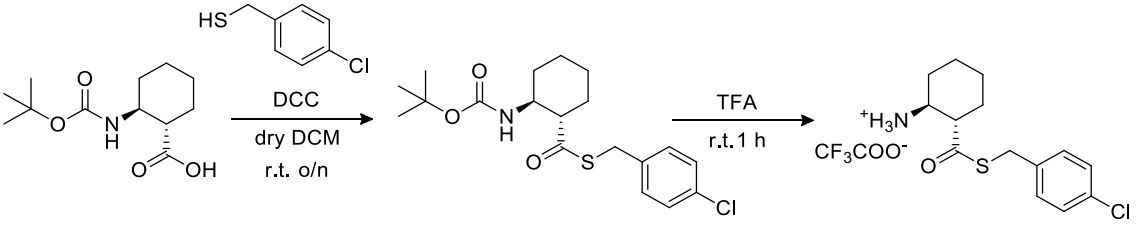


Synthesis of CME ester was performed as previously described.[4] A mixture containing Boc-D-Phg-OH (125.6 mg, 500 µmol), chloroacetonitrile (200 µL), TEA (139.4 µL, 1 mmol) in DMF 200 µL was stirred overnight at room temperature. After reaction, the reaction mixture was diluted with 8 mL Et_2O. The mixture was washed with 1M HCl (6 mL x 2), DW (6 mL x 2), sat. $NaHCO_3$ (6 mL x 2), brine (6 mL x 1). The organic layer was dried over $MgSO_4$, filtered and concentrated. The residual was loaded onto silica gel for purification by flash column chromatography (10:90 → 25:75 → 50:50 → 95:5 EtOAc:n-Hexane gradient elution), yielding the title compound as a white solid (48.1 mg, 33%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.44-7.33 (m, 5H), 5.54-5.19 (m, 2H), 4.84-4.68 (m, 2H), 1.44 (s, 9H); **^{13}C NMR** (101 MHz, $CDCl_3$) δ 170.0, 154.8, 135.0, 129.3, 129.1, 127.3, 113.7, 80.7, 57.6, 49.2, 28.3; **MS** (ESI^+) *m/z*: $[M+Na]^+$ calculated for $C_{15}H_{18}N_2NaO_4^+$; 313.1159, observed; 313.1158.

D-Phg-CME

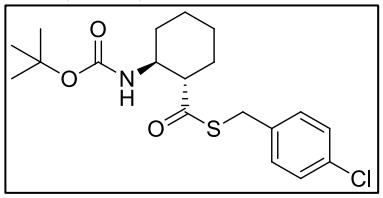


Deprotection of Boc group in the CME ester compound was performed as previously described.[5] To Boc-D-Phg-CME (48.1 mg, 165.8 µmol) in dry ACN 4 mL, 2.5 eq of HCl (103.6 µL of 4M HCl in dioxane) added at 0 °C under nitrogen, and the reaction mixture was stirred for 3 hours at 10-15 °C. After reaction, volatile was evaporated. After evaporation, minimum amount of ACN was added. To the mixture, excess amount of Et_2O was added. The precipitate was filtered, washed with Et_2O and dried under vacuum. The title compound was obtained as a white solid (26.1 mg, 70%). **^{1}H NMR** (400 MHz, DMSO-d_6) δ 9.34 (s, 3H), 7.60-7.42 (m, 5H), 5.43 (s, 1H), 5.18-5.10 (m, 2H); **^{13}C NMR** (101 MHz, DMSO-d_6) δ 167.6, 131.8, 129.8, 129.1, 128.5, 115.2, 55.0, 50.5; **MS** (ESI^+) *m/z*: $[M+H]^+$ calculated for $C_{10}H_{11}N_2O_2^+$; 191.0815, observed; 191.0816.



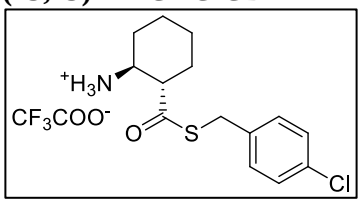
Scheme 9. Synthesis scheme of (1*S*,2*S*)-2-ACHC-CBT.

Boc-(1*S*,2*S*)-2-ACHC-CBT

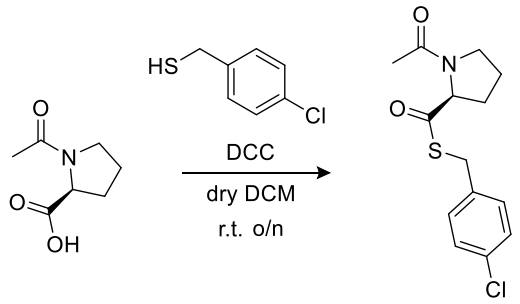


To a solution containing 103.2 mg (500 µmol) of DCC and 66 µL (500 µmol) of 4-chlorobenzyl mercaptan in dry CH_2Cl_2 2 mL, 121.6 mg (500 µmol) of Boc-(1*S*,2*S*)-2-ACHC-OH in dry CH_2Cl_2 1.5 mL was added in a portionwise manner. The mixture was stirred overnight at room temperature. After reaction, the reaction mixture was filtered and concentrated under the reduced pressure. The residual was loaded onto silica gel for purification by flash column chromatography (1:99→ 10:90 → 30:70 → 95:5 EtOAc:n-Hexane gradient elution), yielding the title compound as a white solid (17.3 mg, 9%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.30-7.19 (m, 4H), 4.47-4.26 (m, 1H), 4.07 (s, 2H), 3.78-3.58 (m, 1H), 2.60-2.50 (m, 1H), 2.06-1.90 (m, 2H), 1.75-1.14 (m, 15H); **^{13}C NMR** (101 MHz, $CDCl_3$) δ 200.1, 154.9, 136.4, 133.0, 130.2, 128.7, 79.4, 58.0, 51.6, 33.1, 32.4, 29.6, 28.4, 24.8, 24.6; **MS** (ESI^+) *m/z*: $[M+Na]^+$ calculated for $C_{19}H_{26}ClNNaO_3S^+$; 406.1214, observed; 406.1212.

(1*S*,2*S*)-2-ACHC-CBT

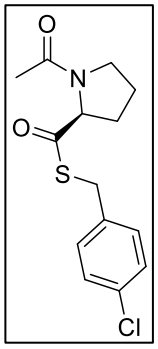


To 17.3 mg of Boc-(1*S*,2*S*)-2-ACHC-CBT, 1 mL of TFA (13.06 mmol) was added, and the mixture was left to stand for 1 hour at room temperature. After reaction, the volatile was evaporated and cold Et_2O was added in the residual. After co-evaporation with Et_2O, the solid was dried under vacuum. The title compound was obtained as a white solid (17.8 mg, 99%). **^{1}H NMR** (400 MHz, DMSO-d_6) δ 8.04 (s, 3H), 7.41-7.30 (m, 4H), 4.23-4.11 (m, 2H), 3.30-3.18 (m, 1H), 2.78-2.72 (m, 1H), 2.02-1.92 (m, 2H), 1.70-1.60 (m, 2H), 1.37-1.22 (m, 4H); **^{13}C NMR** (101 MHz, DMSO-d_6) δ 199.3, 136.3, 131.9, 130.7, 128.5, 54.3, 49.7, 31.7, 29.6, 29.4, 23.9, 23.0; **MS** (ESI^+) *m/z*: $[M+H]^+$ calculated for $C_{14}H_{19}ClNOS^+$; 284.0870, observed; 284.0865.



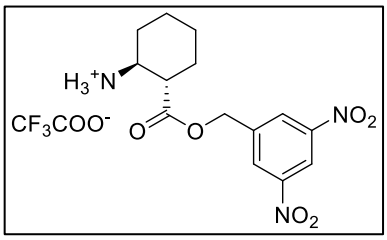
Scheme 10. Synthesis scheme of Ac-L-Pro-CBT.

Ac-L-Pro-CBT



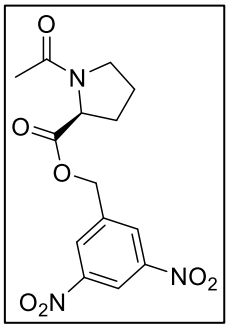
To a solution containing 103.2 mg (500 µmol) of DCC and 66 µL (500 µmol) of 4-chlorobenzyl mercaptan in dry CH_2Cl_2 2 mL, 78.6 mg (500 µmol) of *N*-Acetyl-L-Proline in dry CH_2Cl_2 1.5 mL was added in a portionwise manner. The mixture was stirred overnight at room temperature. After reaction, the reaction mixture was filtered and concentrated under the reduced pressure. The residual was loaded onto silica gel for purification by flash column chromatography (1:99→ 10:90 → 30:70 → 95:5 EtOAc:n-Hexane gradient elution), yielding the title compound as a transparent oily compound (41.2 mg, 28%). **^{1}H NMR** (400 MHz, DMSO-d_6) δ 7.54-7.26 (m, 4H), 4.75-3.94 (m, 3H), 3.57-3.31 (m, 2H), 2.25-1.71 (m, 7H); **^{13}C NMR** (101 MHz, DMSO-d_6) δ 200.3, 169.2, 136.9, 131.7, 130.5, 128.4, 65.1, 47.5, 31.0, 29.8, 24.2, 22.1; **MS** (ESI^+) *m/z*: $[M+Na]^+$ calculated for $C_{14}H_{16}ClNNaO_2S^+$; 320.0482, observed; 320.0478.

(1*S*,2*S*)-2-ACHC-DBE



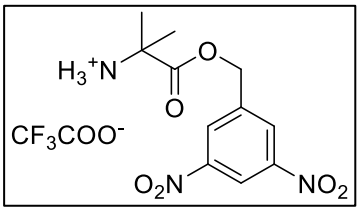
A mixture containing Boc-(1*S*,2*S*)-2-ACHC-OH (121.6 mg, 500 µmol), 3,5-dinitrobenzyl chloride (119 mg, 550 µmol), TEA (77 µL, 550 µmol) in DMF 200 µL was stirred overnight under dark condition at room temperature. After reaction, the reaction mixture was diluted with 20 mL Et_2O. The mixture was washed with 1M HCl (20 mL x 1), sat. $NaHCO_3$ (20 mL x 1), brine (20 mL x 1). The organic layer was dried over $MgSO_4$, filtered and concentrated. The residual was loaded onto silica gel for purification by flash column chromatography (10:90 → 25:75 → 50:50 → 95:5 EtOAc:n-Hexane gradient elution). After evaporation of the eluent, 1 mL of TFA (13.06 mmol) was added, and the mixture was left to stand for 1 hour at room temperature. After reaction, the volatile was evaporated and cold Et_2O was added in the residual. After trituration with Et_2O, the solid was dried under vacuum. The title compound was obtained as a white solid (56 mg, overall yield 26%). **^{1}H NMR** (400 MHz, DMSO-d_6) δ 8.82-8.76 (m, 1H), 8.70-8.62 (m, 2H), 8.20 (s, 3H), 5.38 (s, 2H), 3.29-3.22 (m, 1H), 2.65-2.59 (m, 1H), 2.02-1.95 (m, 2H), 1.73-1.65 (m, 2H), 1.48-1.17 (m, 4H); **^{13}C NMR** (101 MHz, DMSO-d_6) δ 172.3, 148.1, 140.3, 128.4, 118.2, 64.4, 49.9, 46.1, 29.4, 28.2, 23.7, 23.2; **MS** (ESI$^+$) *m/z*: $[M+H]^+$ calculated for $C_{14}H_{18}N_3O_6^+$; 324.1190, observed; 324.1192.

Ac-L-Pro-DBE



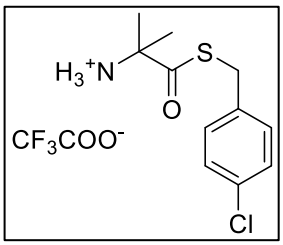
A mixture containing Ac-L-Pro-OH (78.6 mg, 500 µmol), 3,5-dinitrobenzyl chloride (119 mg, 550 µmol), TEA (77 µL, 550 µmol) in DMF 200 µL was stirred overnight under dark condition at room temperature. After reaction, the reaction mixture was diluted with 40 mL Et_2O. The mixture was washed with 1M HCl (20 mL x 1), sat. $NaHCO_3$ (20 mL x 1), brine (20 mL x 1). The organic layer was dried over $MgSO_4$, filtered and concentrated. The residual was loaded onto silica gel for purification by flash column chromatography (10:90 → 25:75 → 50:50 → 95:5 EtOAc:n-Hexane gradient elution), yielding the title compound as a white solid (69.4 mg, 41%). **^{1}H NMR** (400 MHz, DMSO-d_6) δ 8.80-8.76 (m, 1H), 8.68-8.59 (m, 2H), 5.47-5.34 (m, 2H), 4.39-4.34 (m, 1H), 3.60-3.32 (m, 2H), 2.33-1.78 (m, 7H); **^{13}C NMR** (101 MHz, DMSO-d_6) δ 171.8, 168.6, 148.1, 140.7, 127.6, 117.9, 63.7, 58.2, 47.3, 29.0, 24.6, 21.9; **MS** (ESI$^+$) *m/z*: $[M+Na]^+$ calculated for $C_{14}H_{15}N_3NaO_7^+$; 360.0802, observed; 360.0797.

Aib-DBE

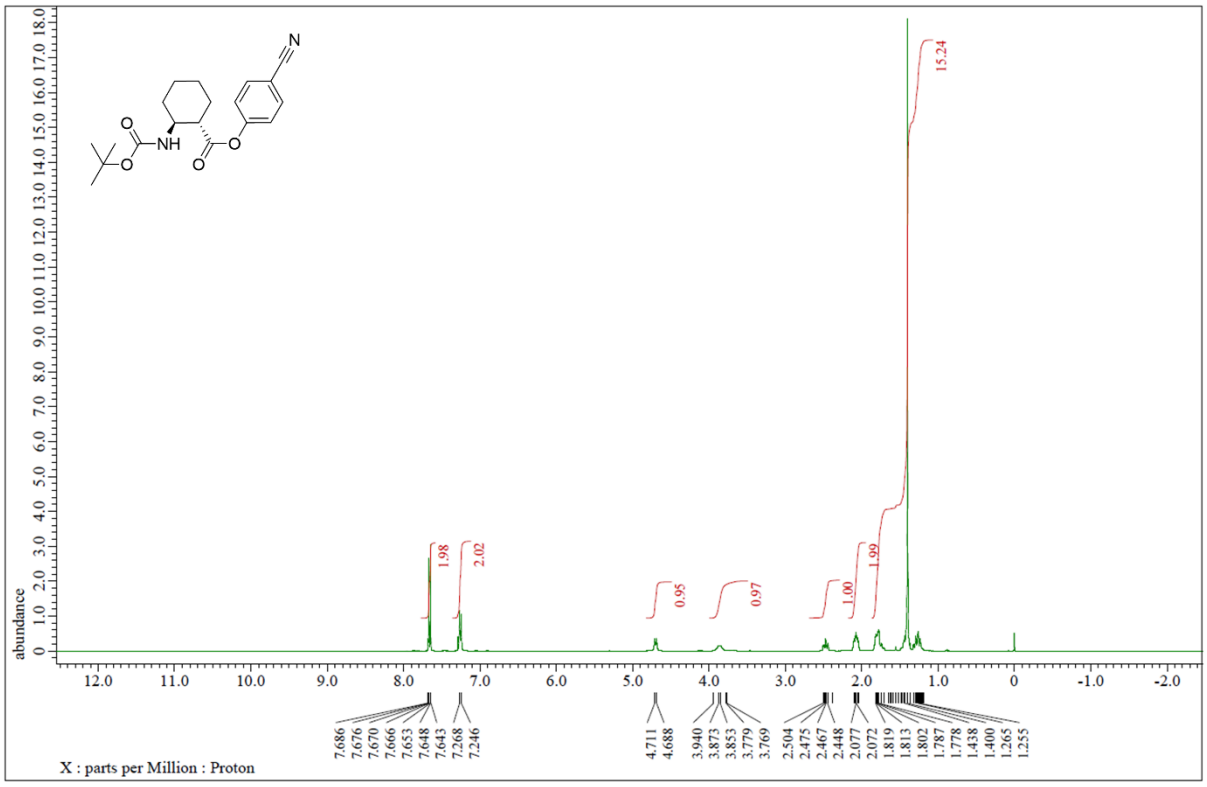


A mixture containing Boc-Aib-OH (203.2 mg, 1 mmol), 3,5-dinitrobenzyl chloride (238.2 mg, 1.1 mmol), TEA (154 µL, 1.1 mmol) in DMF 400 µL was stirred overnight under dark condition at room temperature. After reaction, the reaction mixture was diluted with 40 mL Et_2O. The mixture was washed with 1M HCl (40 mL x 1), sat. $NaHCO_3$ (40 mL x 1), brine (40 mL x 1). The organic layer was dried over $MgSO_4$, filtered and concentrated. The residual was loaded onto silica gel for purification by flash column chromatography (10:90 → 25:75 → 50:50 → 95:5 EtOAc:n-Hexane gradient elution). After evaporation of the eluent, 2 mL of TFA (26.12 mmol) was added, and the mixture was left to stand for 1 hour at room temperature. After reaction, the volatile was evaporated. The remained TFA was removed by repeating addition of Et_2O and concentration under reduced pressure. After trituration with Et_2O, the solid was dried under vacuum. The title compound was obtained as a white solid (200 mg, overall yield 50%). **^{1}H NMR** (400 MHz, DMSO-d_6) δ 8.87-8.73 (m, 6H), 5.49 (s, 2H), 1.55 (s, 6H); **^{13}C NMR** (101 MHz, DMSO-d_6) δ 171.3, 148.2, 139.9, 128.5, 118.4, 65.3, 56.0, 23.5; **MS** (ESI^+) *m/z*: $[M+H]^+$ calculated for $C_{11}H_{14}N_3O_6^+$; 284.0877, observed; 284.0879.

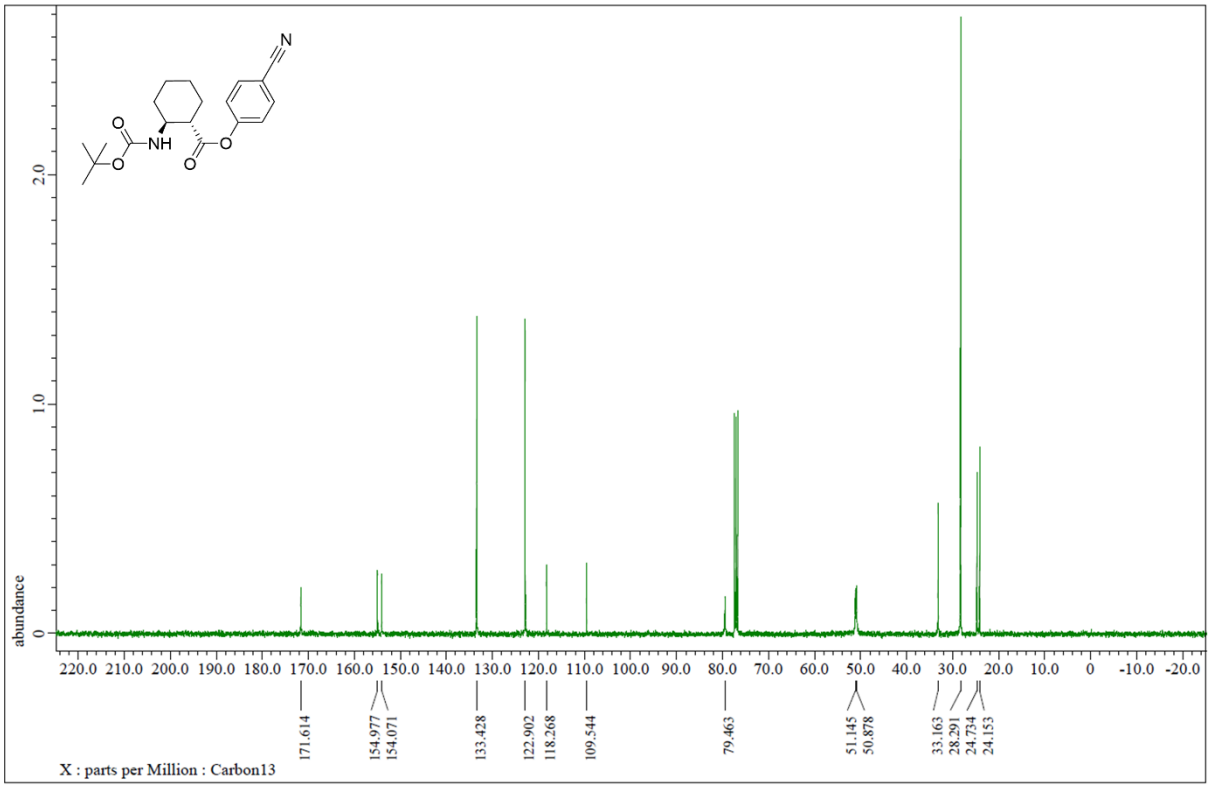
Aib-CBT



To a solution containing 206.3 mg (1 mmol) of DCC and 132 µL (1 mmol) of 4-chlorobenzyl mercaptan in dry CH_2Cl_2 4 mL, 203.2 mg (1 mmol) of Boc-Aib-OH in dry CH_2Cl_2 3 mL was added in a portionwise manner. The mixture was stirred overnight at room temperature. After reaction, the reaction mixture was filtered and concentrated under the reduced pressure. The residual was loaded onto silica gel for purification by flash column chromatography (1:99→ 10:90 → 30:70 → 95:5 EtOAc:n-Hexane gradient elution). After evaporation of the eluent, 2 mL of TFA (26.12 mmol) was added, and the mixture was left to stand for 1 hour at room temperature. After reaction, the volatile was evaporated. The remained TFA was removed by repeating addition of Et_2O and concentration under reduced pressure. After trituration with Et_2O, the solid was dried under vacuum. The title compound was obtained as a white solid (221 mg, overall yield 62%). **^{1}H NMR** (400 MHz, DMSO-d_6) δ 8.81 (s, 3H), 7.47-7.26 (m, 4H), 4.20 (s, 2H), 1.52 (s, 6H); **^{13}C NMR** (101 MHz, DMSO-d_6) δ 199.7, 136.1, 132.2, 130.7, 128.6, 61.8, 31.7, 24.0; **MS** (ESI^+) *m/z*: $[M+H]^+$ calculated for $C_{11}H_{15}ClNOS^+$; 244.0557, observed; 244.0557.

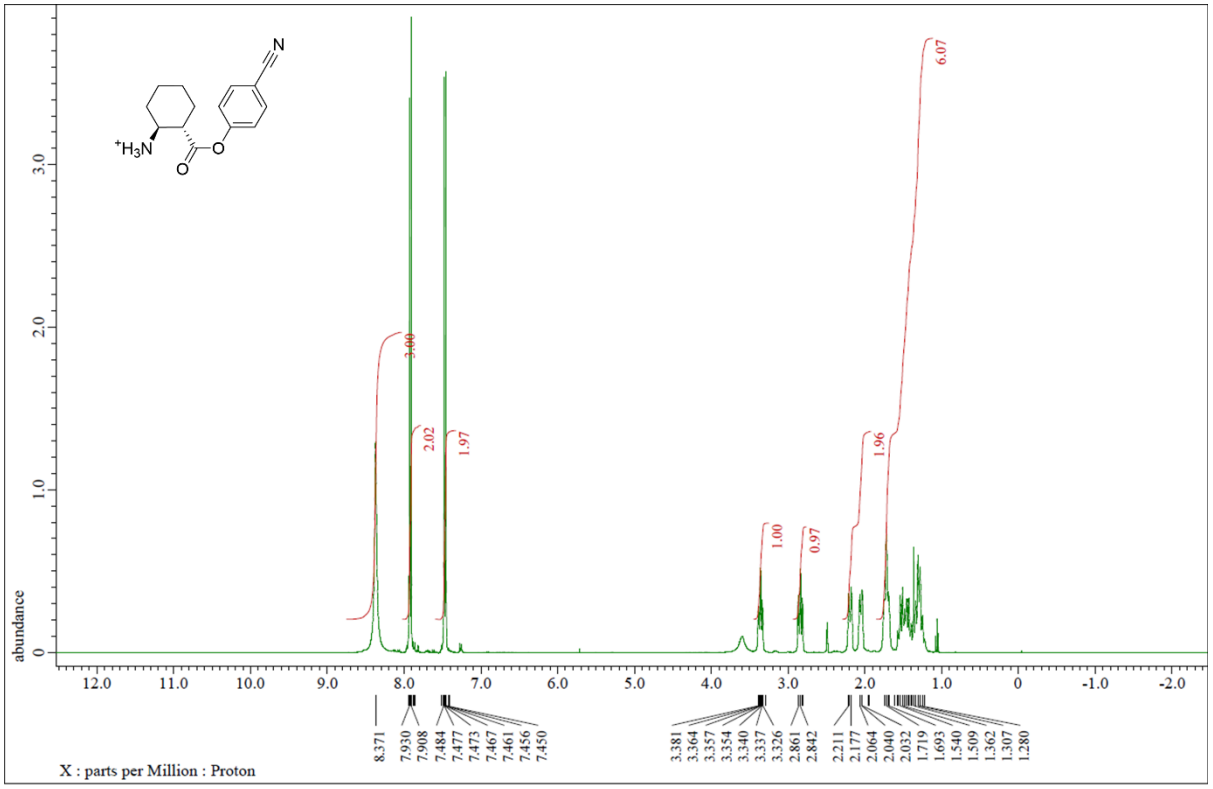


^{1}H NMR (400 MHz, $CDCl_3$) δ 7.69-7.64 (m, 2H), 7.26 (d, J = 8.7 Hz, 2H), 4.70 (d, J = 9.2 Hz, 1H), 3.94-3.77 (m, 1H), 2.50-2.39 (m, 1H), 2.10-2.04 (m, 2H), 1.82-1.19 (m, 15H)

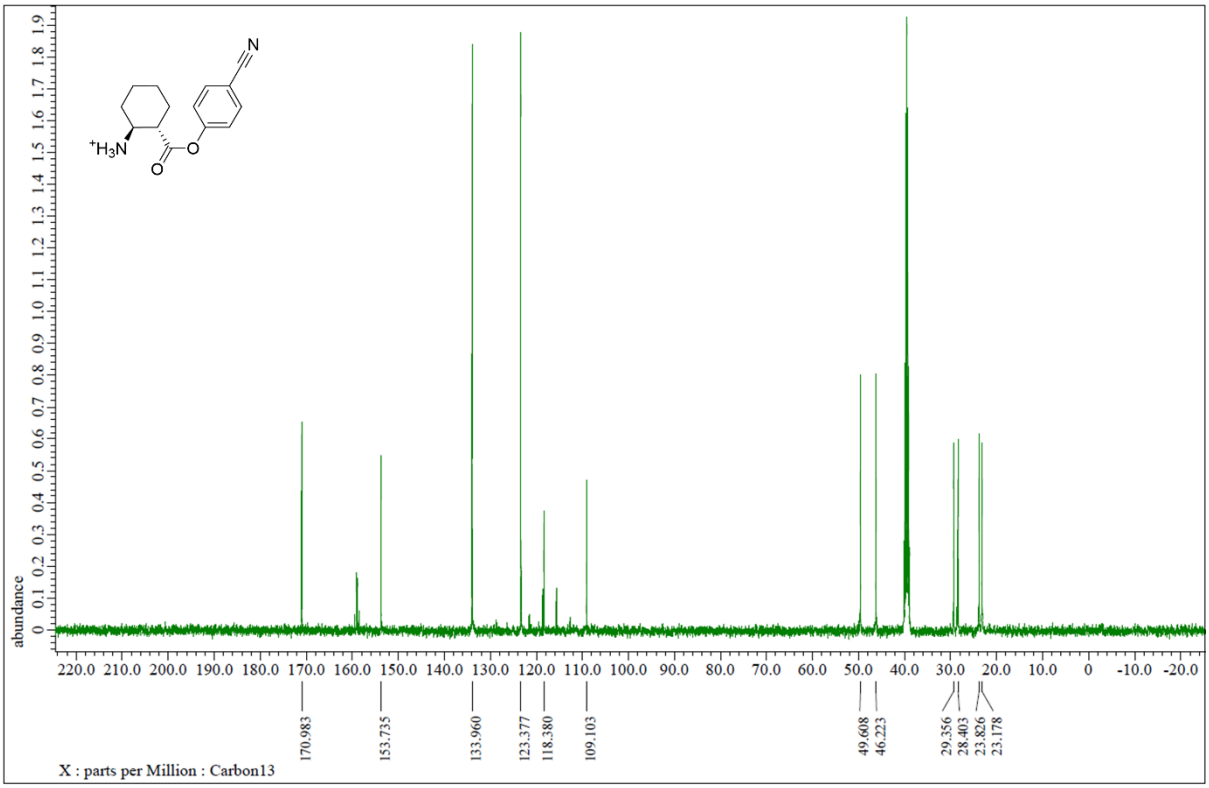


^{13}C NMR (101 MHz, $CDCl_3$) δ 171.6, 155.0, 154.1, 133.4, 122.9, 118.3, 109.5, 79.5, 51.1, 50.9, 33.2, 28.3, 24.7, 24.2

Supplementary Fig. 16. ^{1}H NMR and ^{13}C NMR spectra of Boc-(1*S*,2*S*)-2-ACHC-4CP

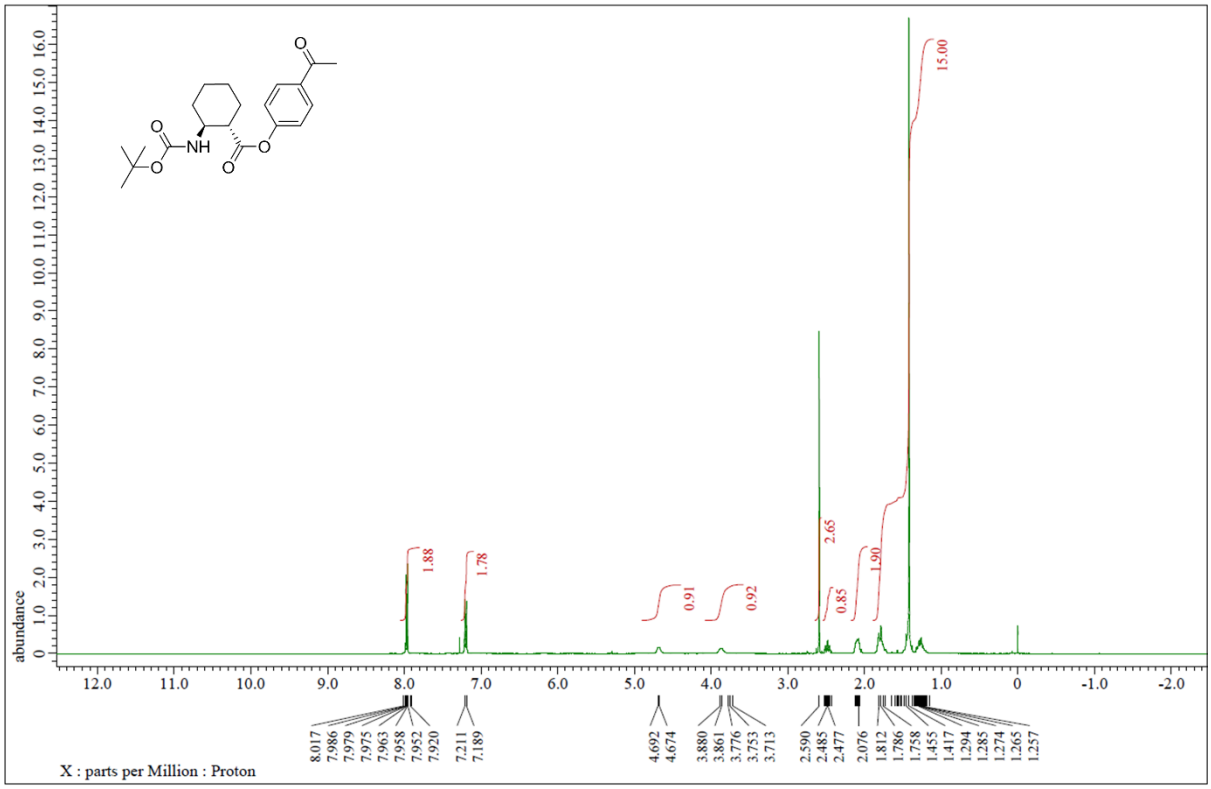


^{1}H NMR (400 MHz, DMSO-d_6) δ 8.37 (s, 3H), 7.95-7.86 (m, 2H), 7.52-7.40 (m, 2H), 3.39-3.29 (m, 1H), 2.87-2.81 (m, 1H), 2.21-1.94 (m, 2H), 1.74-1.22 (m, 6H)

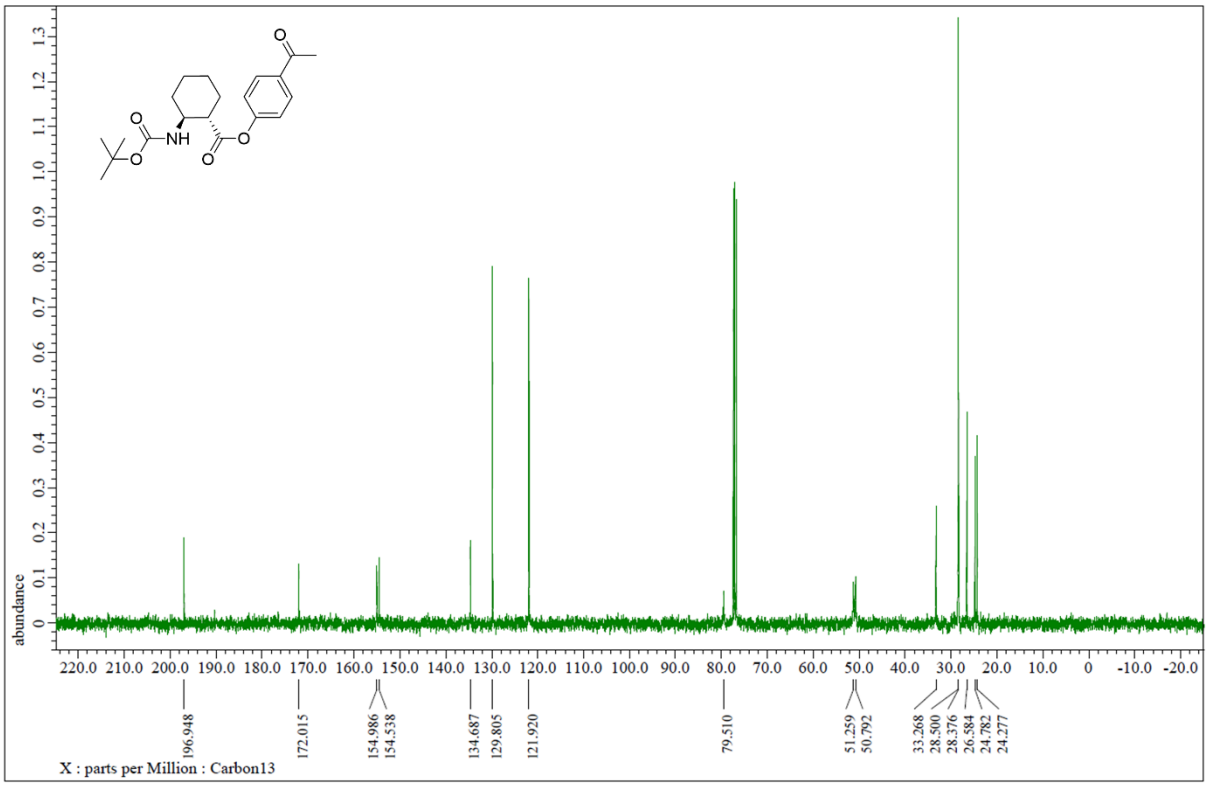


^{13}C NMR (101 MHz, DMSO-d_6) δ 171.0, 153.7, 134.0, 123.4, 118.4, 109.1, 49.6, 46.2, 29.4, 28.4, 23.8, 23.2

Supplementary Fig. 17. ^{1}H NMR and ^{13}C NMR spectra of (1*S*,2*S*)-2-ACHC-4CP

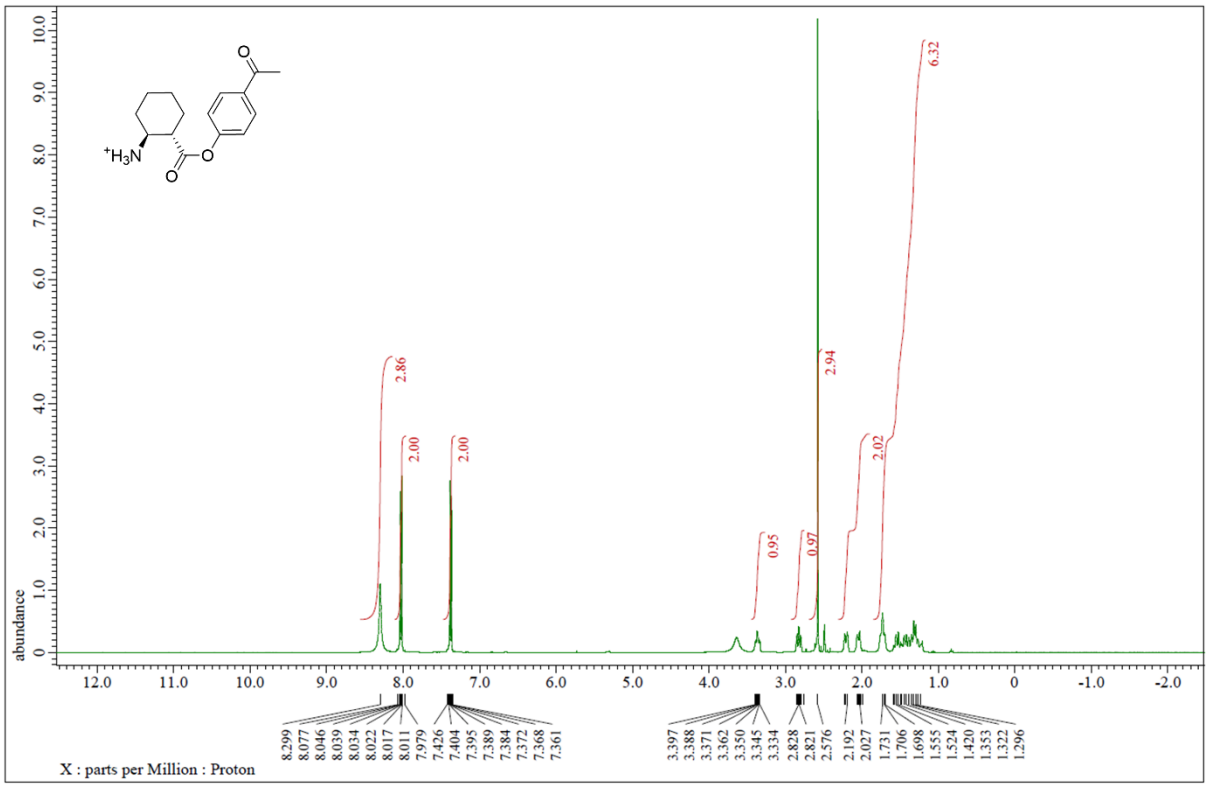


^{1}H NMR (400 MHz, $CDCl_3$) δ 8.02-7.91 (m, 2H), 7.20 (d, J = 8.7 Hz, 2H), 4.68 (d, J = 7.3 Hz, 1H), 3.88-3.71 (m, 1H), 2.59 (s, 3H), 2.53-2.43 (m, 1H), 2.12-2.07 (m, 2H), 1.81-1.16 (m, 15H)

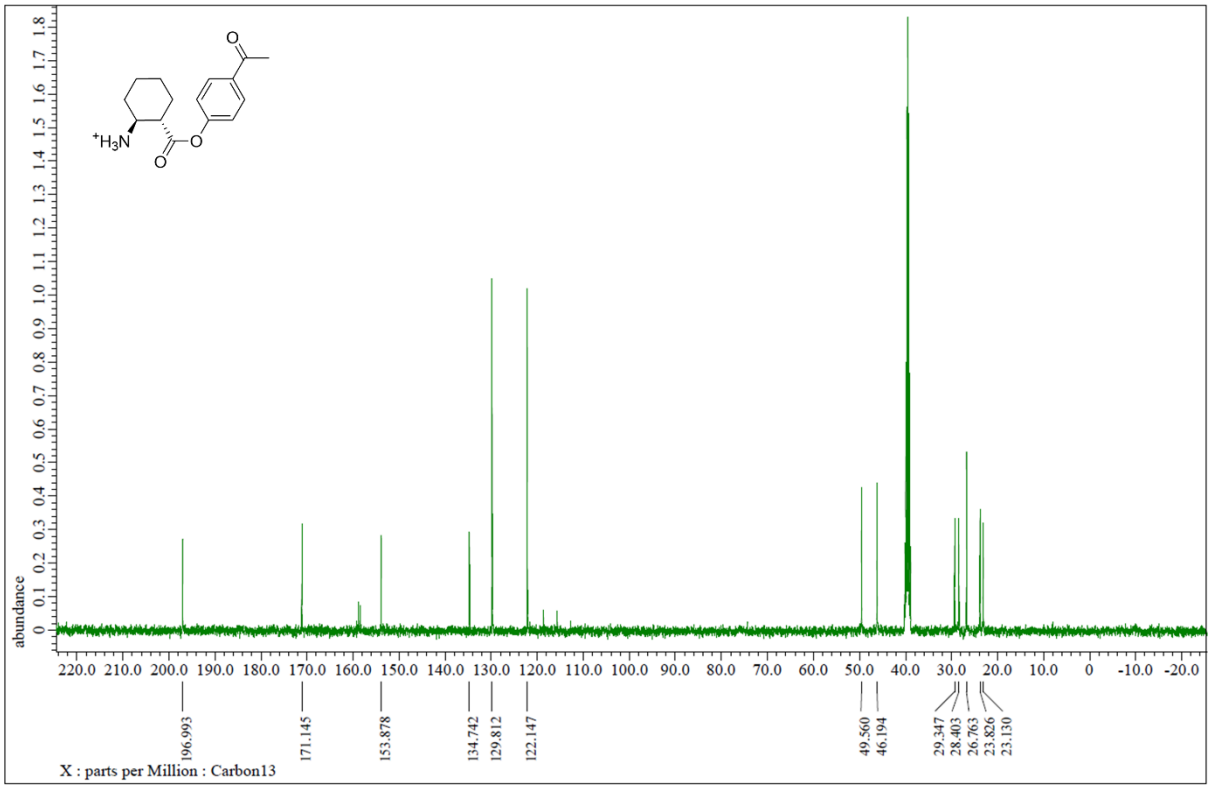


^{13}C NMR (101 MHz, $CDCl_3$) δ 196.9, 172.0, 155.0, 154.5, 134.7, 129.8, 121.9, 79.5, 51.3, 50.8, 33.3, 28.5, 28.4, 26.6, 24.8, 24.3

Supplementary Fig. 18. ^{1}H NMR and ^{13}C NMR spectra of Boc-(1*S*,2*S*)-2-ACHC-4AP

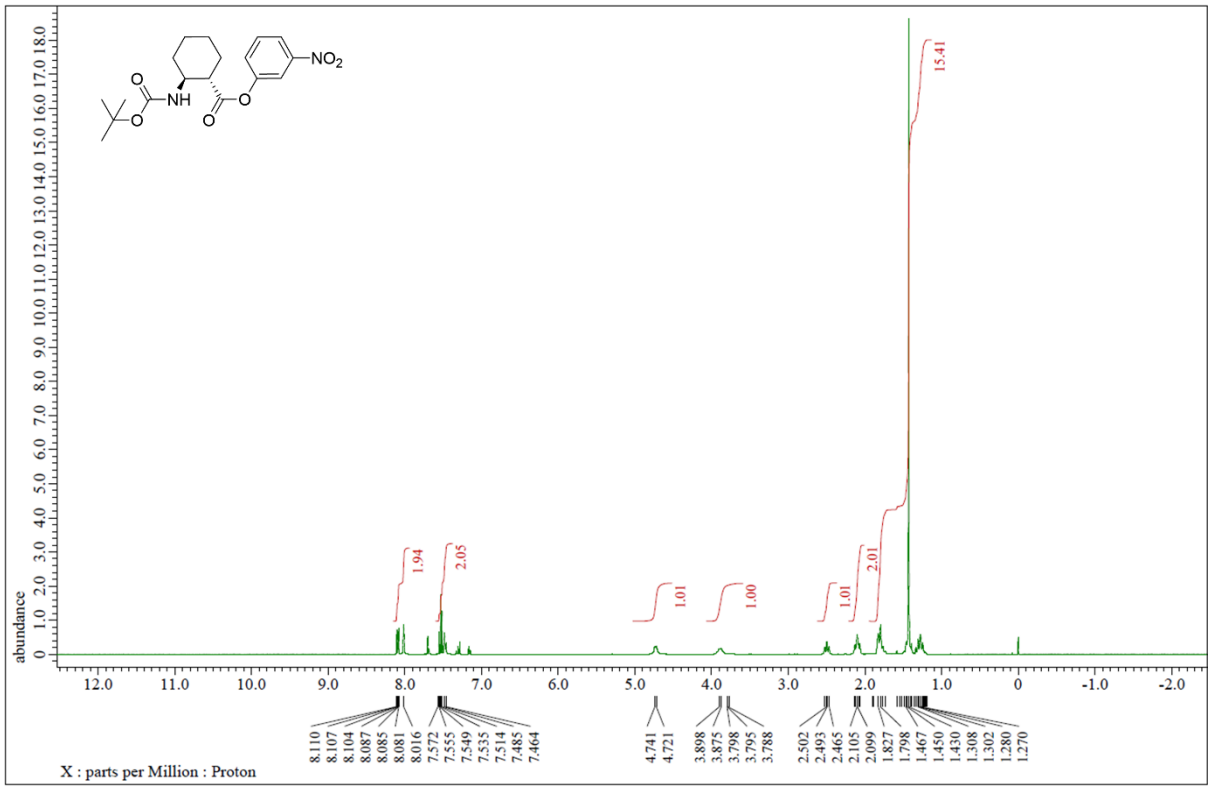


^{1}H NMR (400 MHz, DMSO-d_6) δ 8.30 (s, 3H), 8.08-7.98 (m, 2H), 7.43-7.35 (m, 2H), 3.40-3.33 (m, 1H), 2.86-2.76 (m, 1H), 2.58 (s, 3H), 2.23-1.99 (m, 2H), 1.73-1.23 (m, 6H)

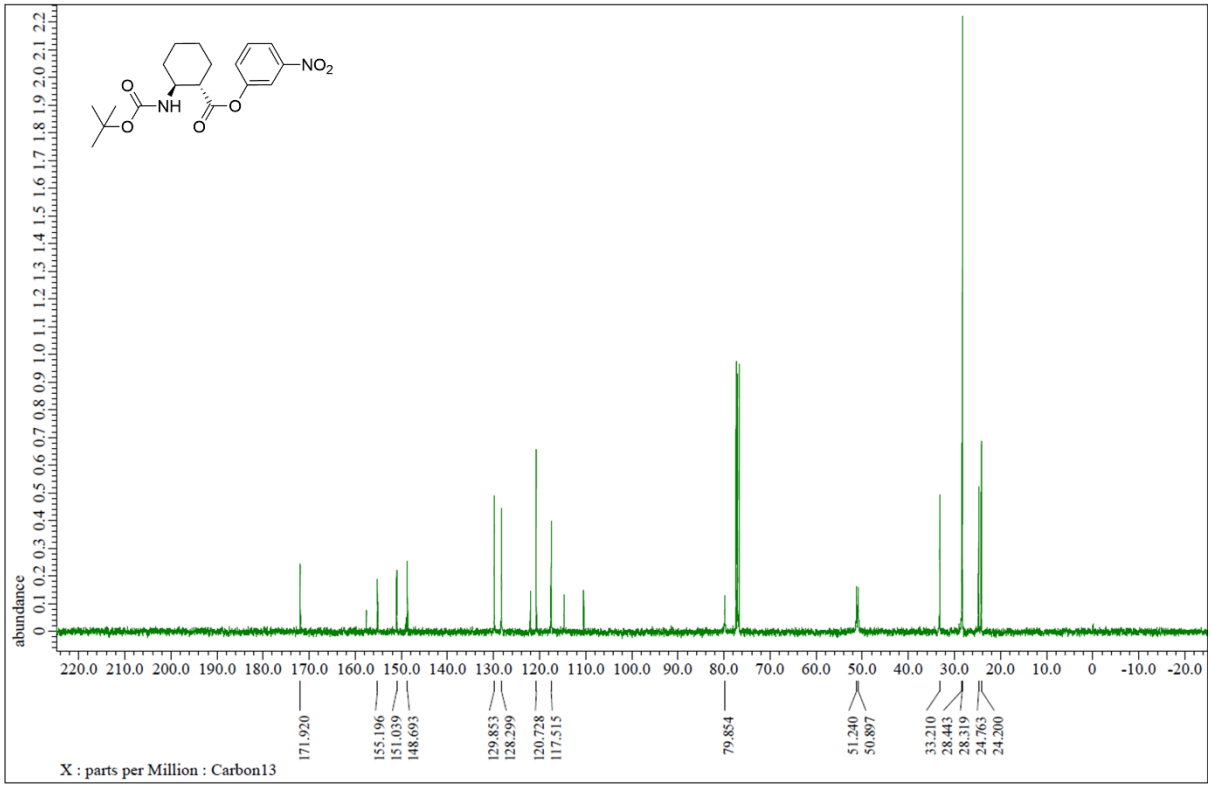


^{13}C NMR (101 MHz, DMSO-d_6) δ 197.0, 171.1, 153.9, 134.7, 129.8, 122.1, 49.6, 46.2, 29.3, 28.4, 26.8, 23.8, 23.1

Supplementary Fig. 19. ^{1}H NMR and ^{13}C NMR spectra of (1*S*,2*S*)-2-ACHC-4AP

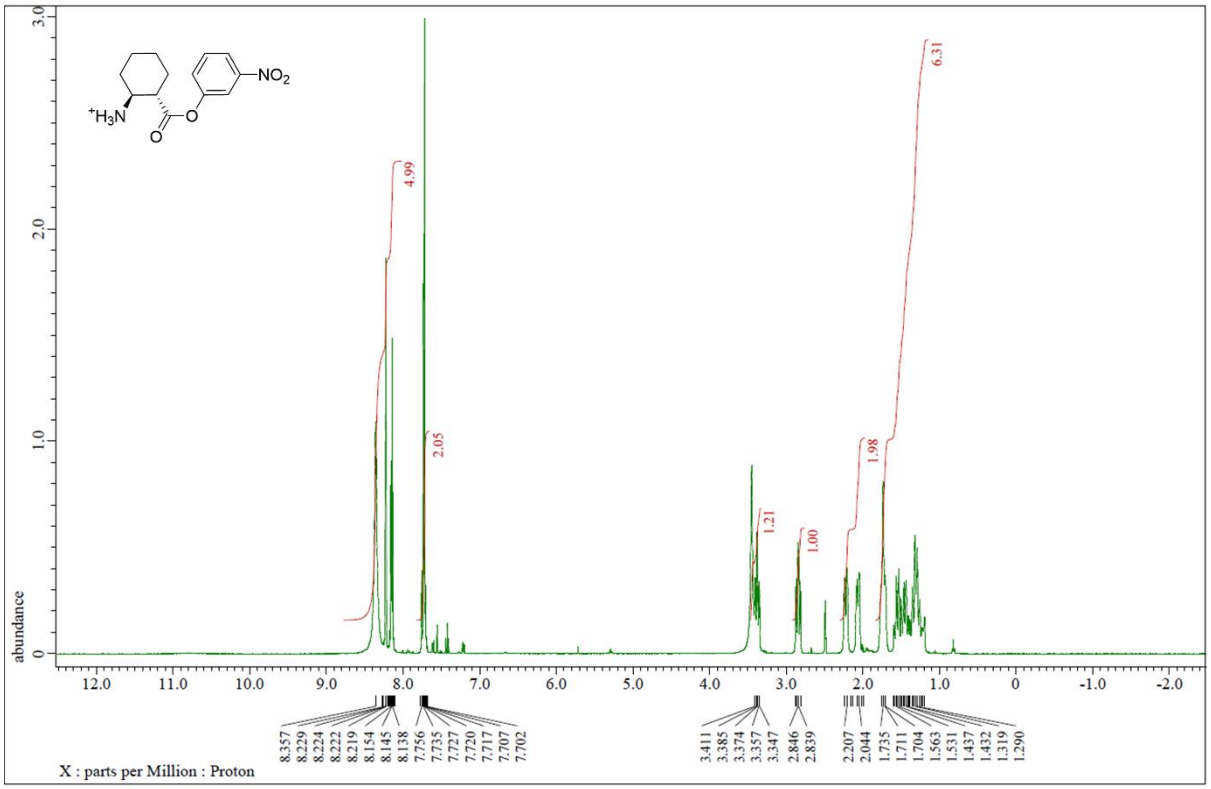


^{1}H NMR (400 MHz, $CDCl_3$) δ 8.11-8.02 (m, 2H), 7.57-7.46 (m, 2H), 4.73 (d, J = 8.2 Hz, 1H), 3.90-3.77 (m, 1H), 2.53-2.47 (m, 1H), 2.14-2.07 (m, 2H), 1.90-1.20 (m, 15H)

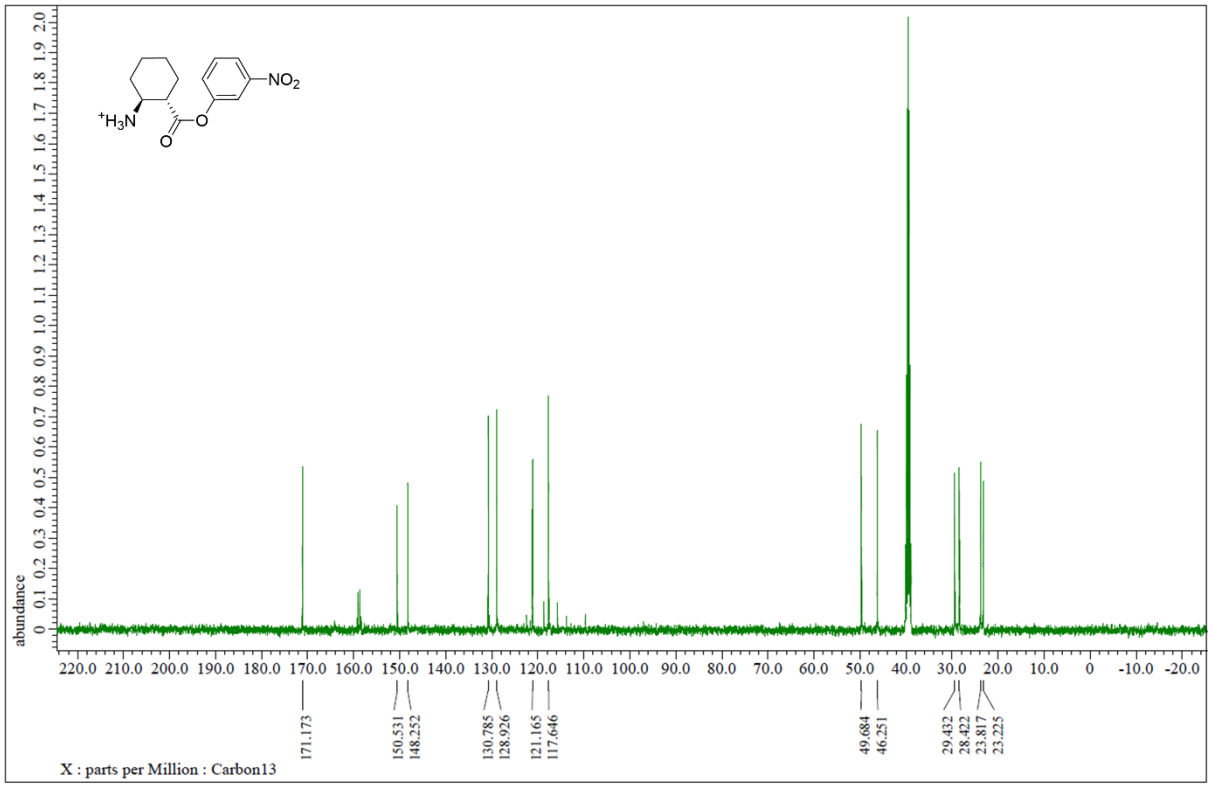


^{13}C NMR (101 MHz, $CDCl_3$) δ 171.9, 155.2, 151.0, 148.7, 129.9, 128.3, 120.7, 117.5, 79.9, 51.2, 50.9, 33.2, 28.4, 28.3, 24.8, 24.2

Supplementary Fig. 20. ^{1}H NMR and ^{13}C NMR spectra of Boc-(1*S*,2*S*)-2-ACHC-3NP

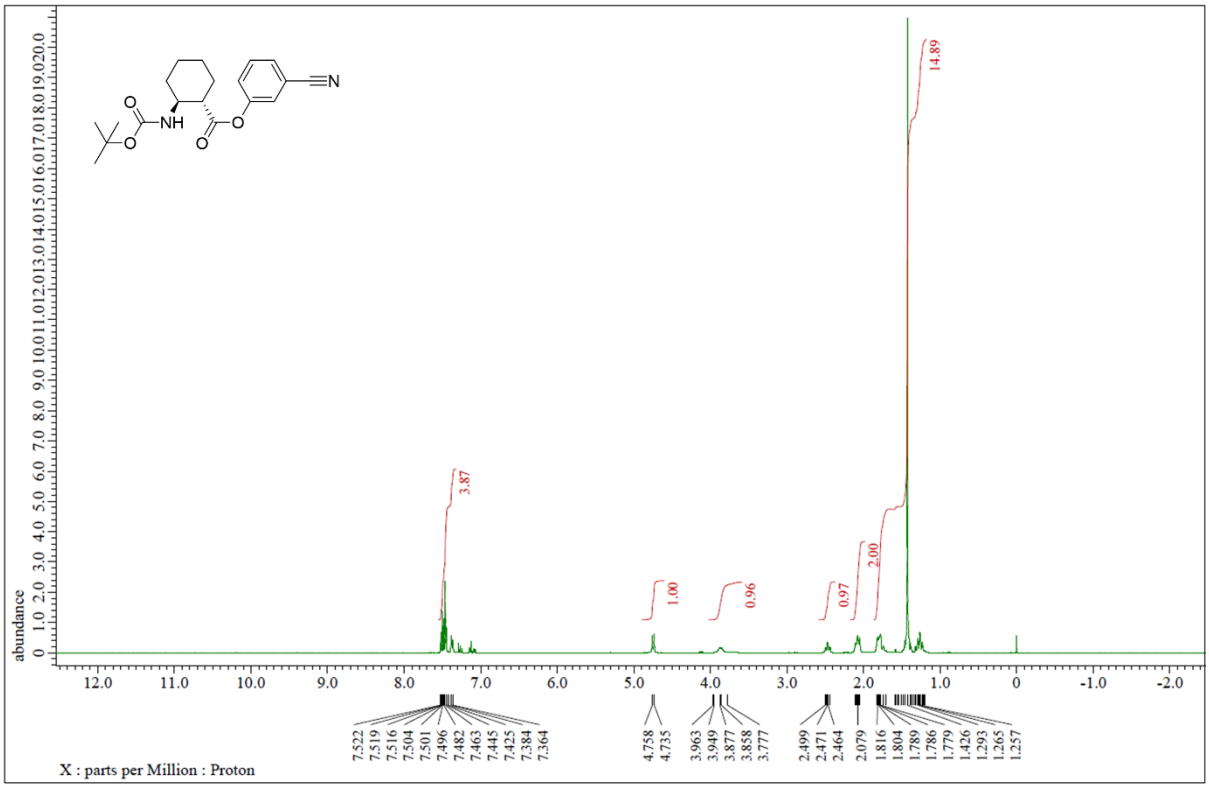


^{1}H NMR (400 MHz, DMSO-d_6) δ 8.36-8.11 (m, 5H), 7.77-7.68 (m, 2H), 3.41-3.35 (m, 1H), 2.88-2.81 (m, 1H), 2.24-1.99 (m, 2H), 1.76-1.19 (m, 6H)

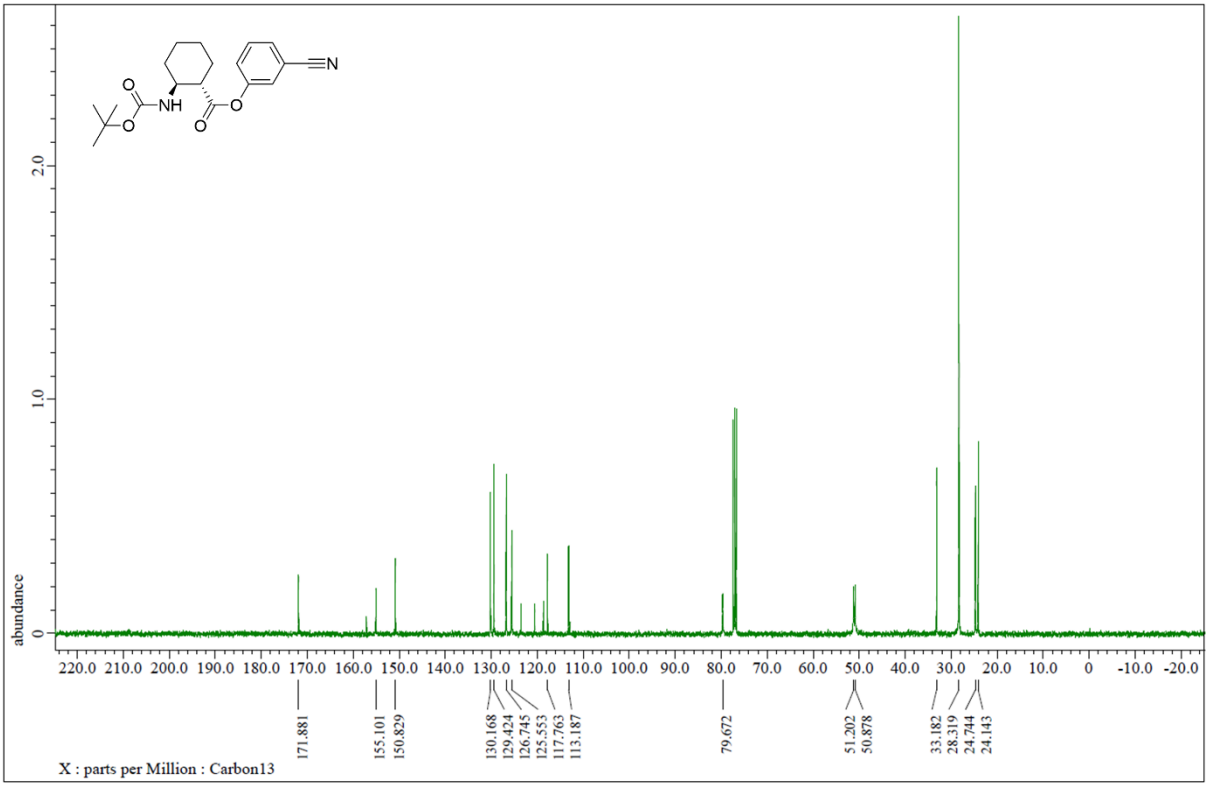


^{13}C NMR (101 MHz, DMSO-d_6) δ 171.2, 150.5, 148.3, 130.8, 128.9, 121.2, 117.6, 49.7, 46.3, 29.4, 28.4, 23.8, 23.2

Supplementary Fig. 21. ^{1}H NMR and ^{13}C NMR spectra of (1*S*,2*S*)-2-ACHC-3NP

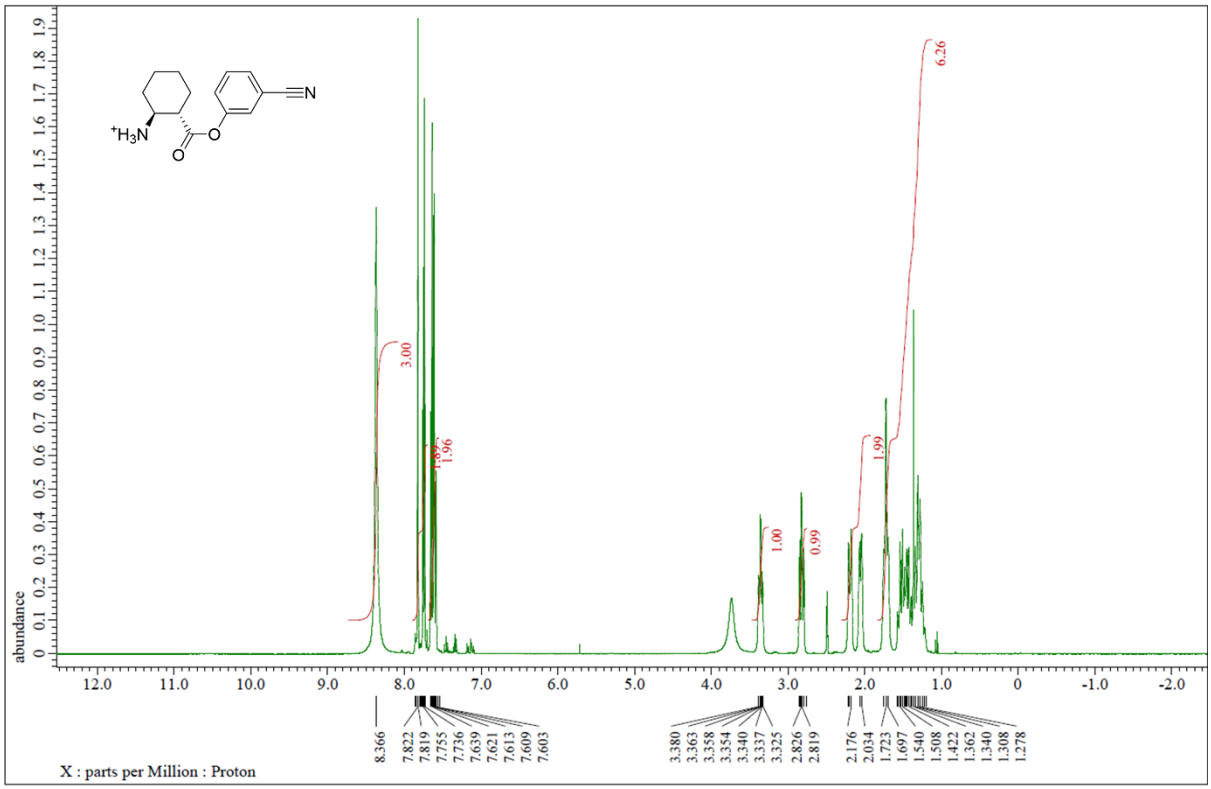


^{1}H NMR (400 MHz, $CDCl_3$) δ 7.52-7.36 (m, 4H), 4.75 (d, J = 9.2 Hz, 1H), 3.96-3.78 (m, 1H), 2.50-2.44 (m, 1H), 2.11-2.04 (m, 2H), 1.82-1.20 (m, 15H)

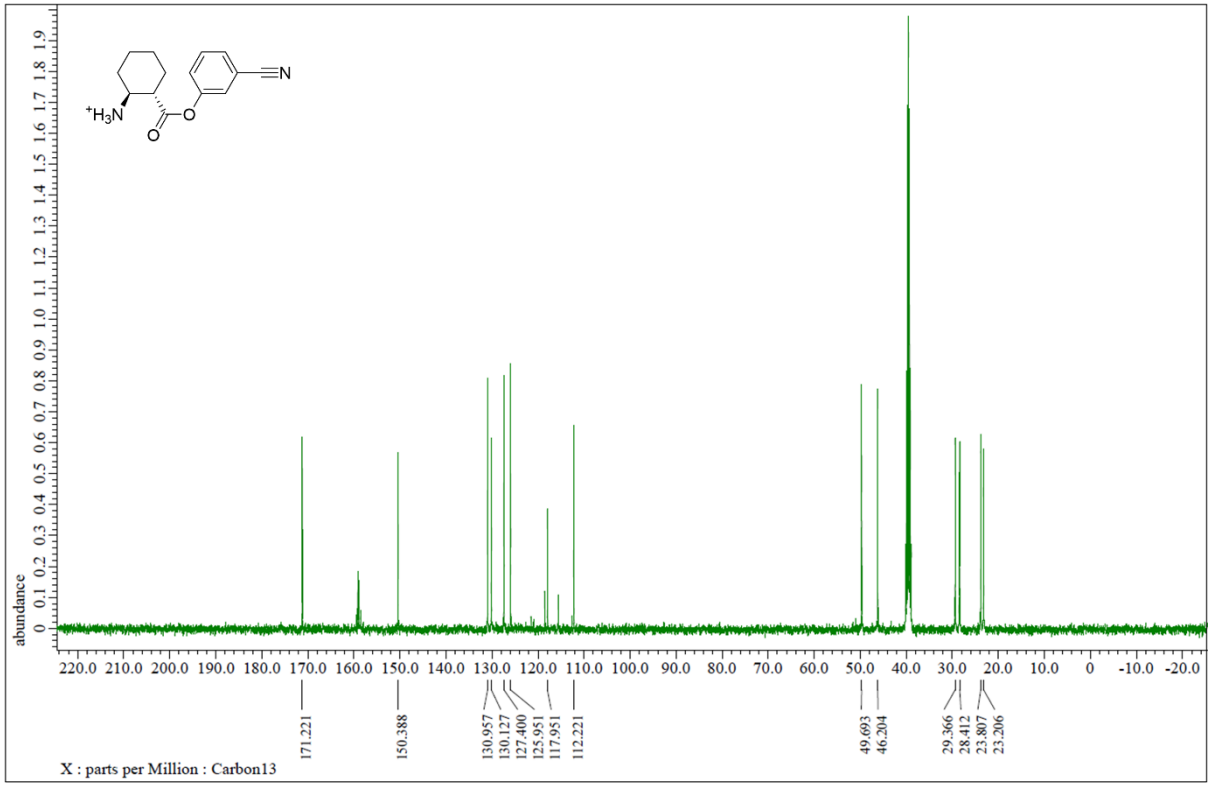


^{13}C NMR (101 MHz, $CDCl_3$) δ 171.9, 155.1, 150.8, 130.2, 129.4, 126.7, 125.6, 117.8, 113.2, 79.7, 51.2, 50.9, 33.2, 28.3, 24.7, 24.1

Supplementary Fig. 22. ^{1}H NMR and ^{13}C NMR spectra of Boc-(1*S*,2*S*)-2-ACHC-3CP

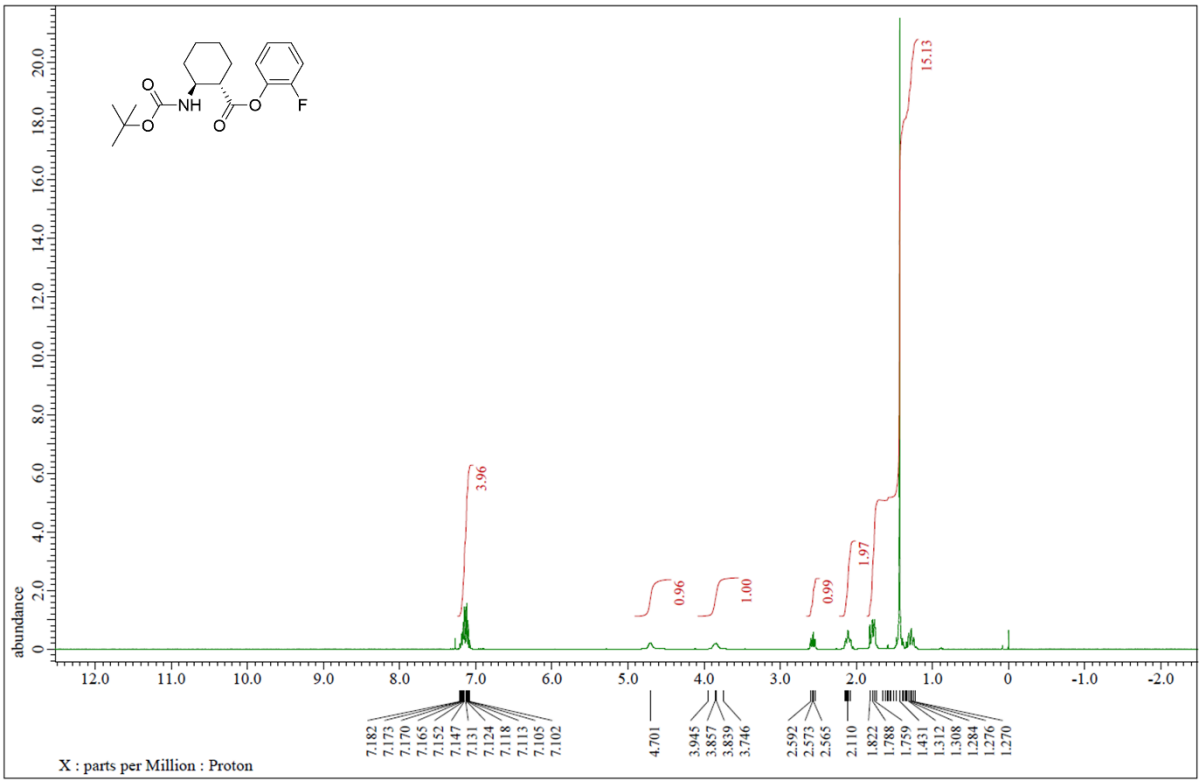


^{1}H NMR (400 MHz, DMSO-d_6) δ 8.37 (s, 3H), 7.86-7.73 (m, 2H), 7.66-7.56 (m, 2H), 3.39-3.33 (m, 1H), 2.85-2.76 (m, 1H), 2.21-2.03 (m, 2H), 1.75-1.20 (m, 6H)

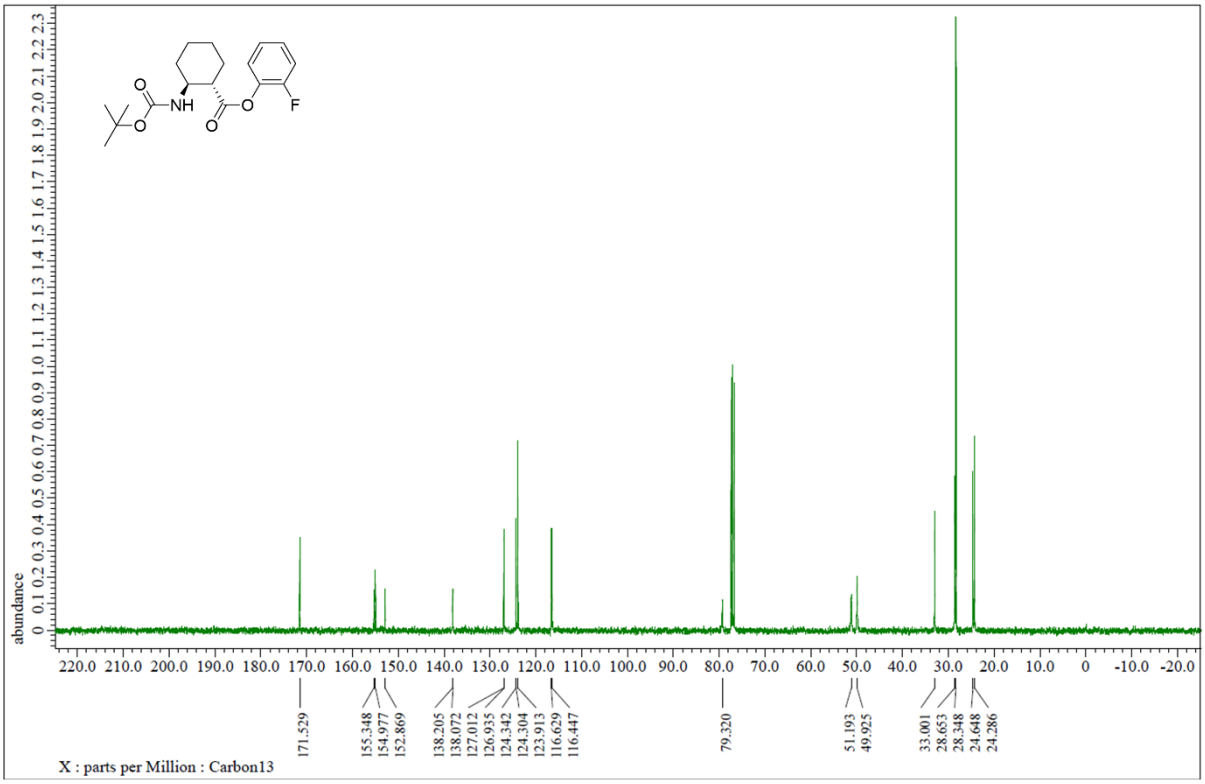


^{13}C NMR (101 MHz, DMSO-d_6) δ 171.2, 150.4, 131.0, 130.1, 127.4, 126.0, 118.0, 112.2, 49.7, 46.2, 29.4, 28.4, 23.8, 23.2

Supplementary Fig. 23. ^{1}H NMR and ^{13}C NMR spectra of (1*S*,2*S*)-2-ACHC-3CP

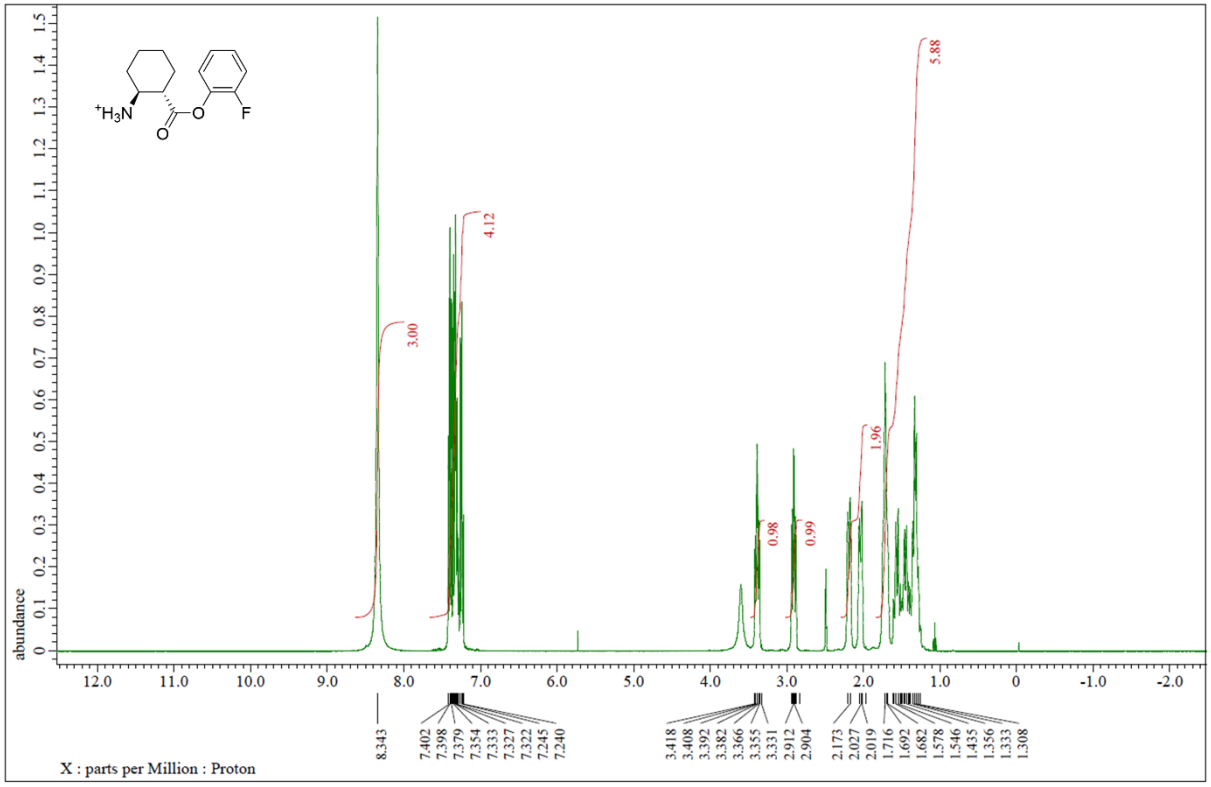


^{1}H NMR (400 MHz, $CDCl_3$) δ 7.21-7.08 (m, 4H), 4.70 (s, 1H), 3.95-3.75 (m, 1H), 2.60-2.54 (m, 1H), 2.15-2.07 (m, 2H), 1.82-1.22 (m, 15H)

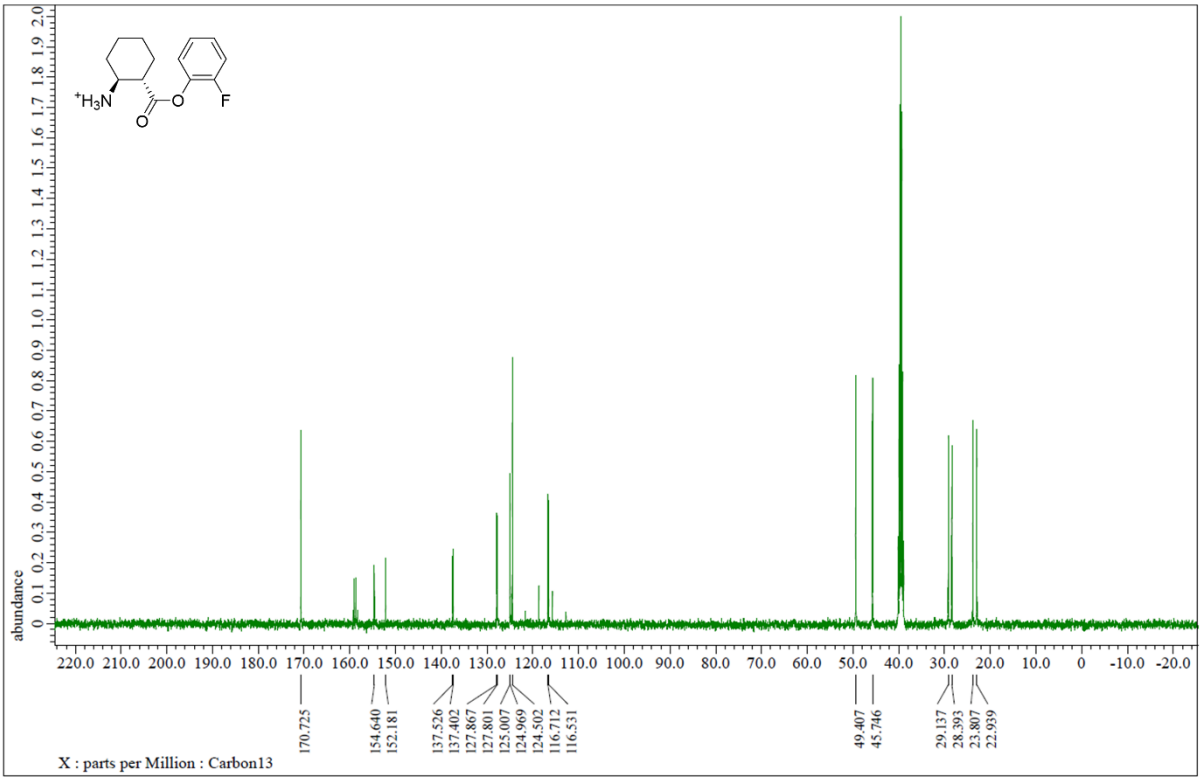


^{13}C NMR (101 MHz, $CDCl_3$) δ 171.5, 155.0, 154.1 (d, J = 249.2 Hz), 138.1 (d, J = 13.4 Hz), 127.0 (d, J = 7.7 Hz), 124.3 (d, J = 3.8 Hz), 123.9, 116.5 (d, J = 18.2 Hz), 79.3, 51.2, 49.9, 33.0, 28.7, 28.3, 24.6, 24.3

Supplementary Fig. 24. ^{1}H NMR and ^{13}C NMR spectra of Boc-(1*S*,2*S*)-2-ACHC-2FP

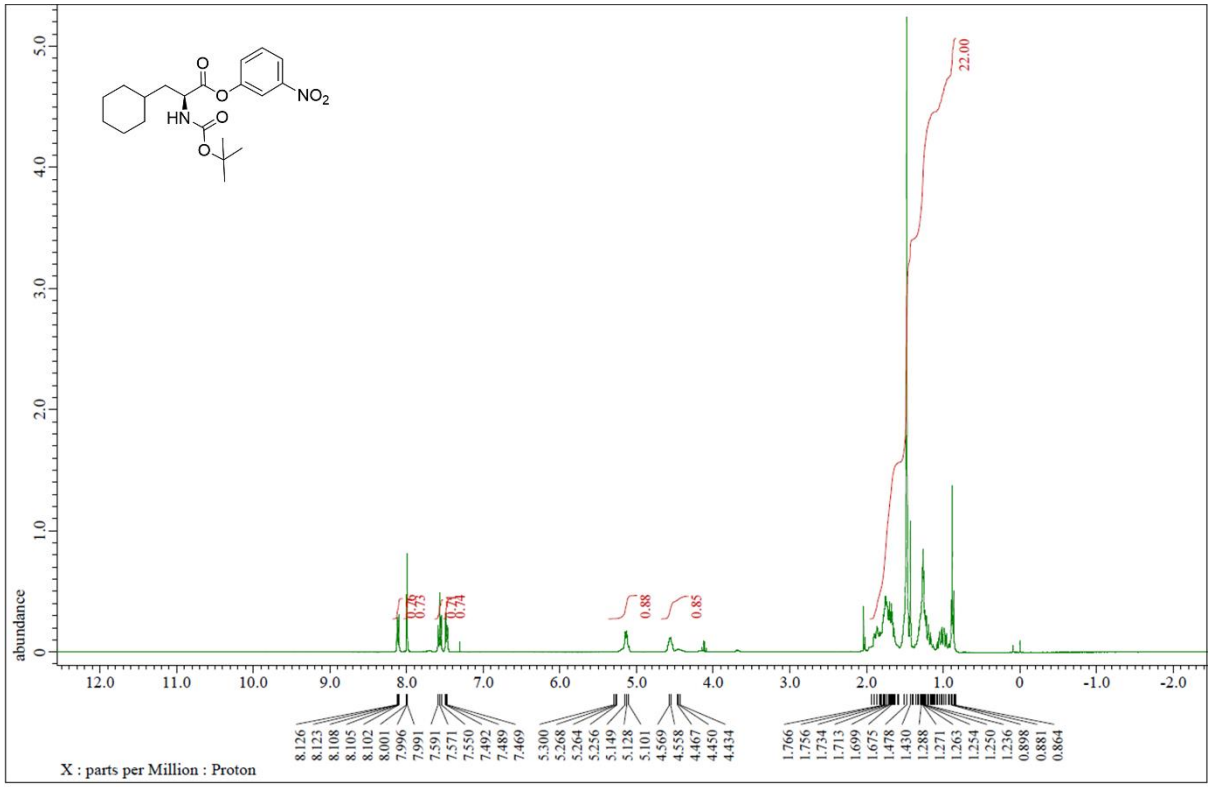


^{1}H NMR (400 MHz, DMSO-d_6) δ 8.34 (s, 3H), 7.42-7.22 (m, 4H), 3.42-3.33 (m, 1H), 2.94-2.84 (m, 1H), 2.21-1.96 (m, 2H), 1.72-1.25 (m, 6H)

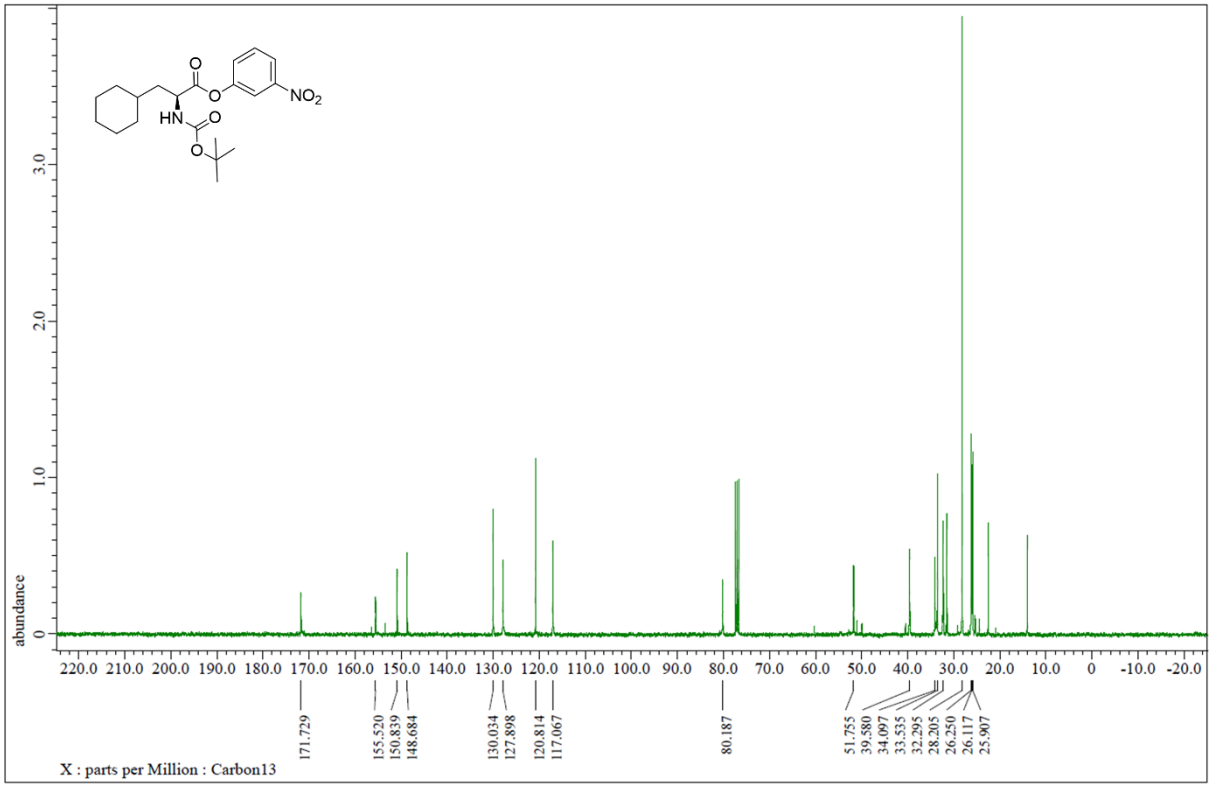


^{13}C NMR (101 MHz, DMSO-d_6) δ 170.7, 153.4 (d, J = 247.3 Hz), 137.5 (d, J = 12.5 Hz), 127.8 (d, J = 6.7 Hz), 125.0 (d, J = 3.8 Hz), 124.5, 116.6 (d, J = 18.2 Hz), 49.4, 45.7, 29.1, 28.4, 23.8, 22.9

Supplementary Fig. 25. ^{1}H NMR and ^{13}C NMR spectra of (1*S*,2*S*)-2-ACHC-2FP

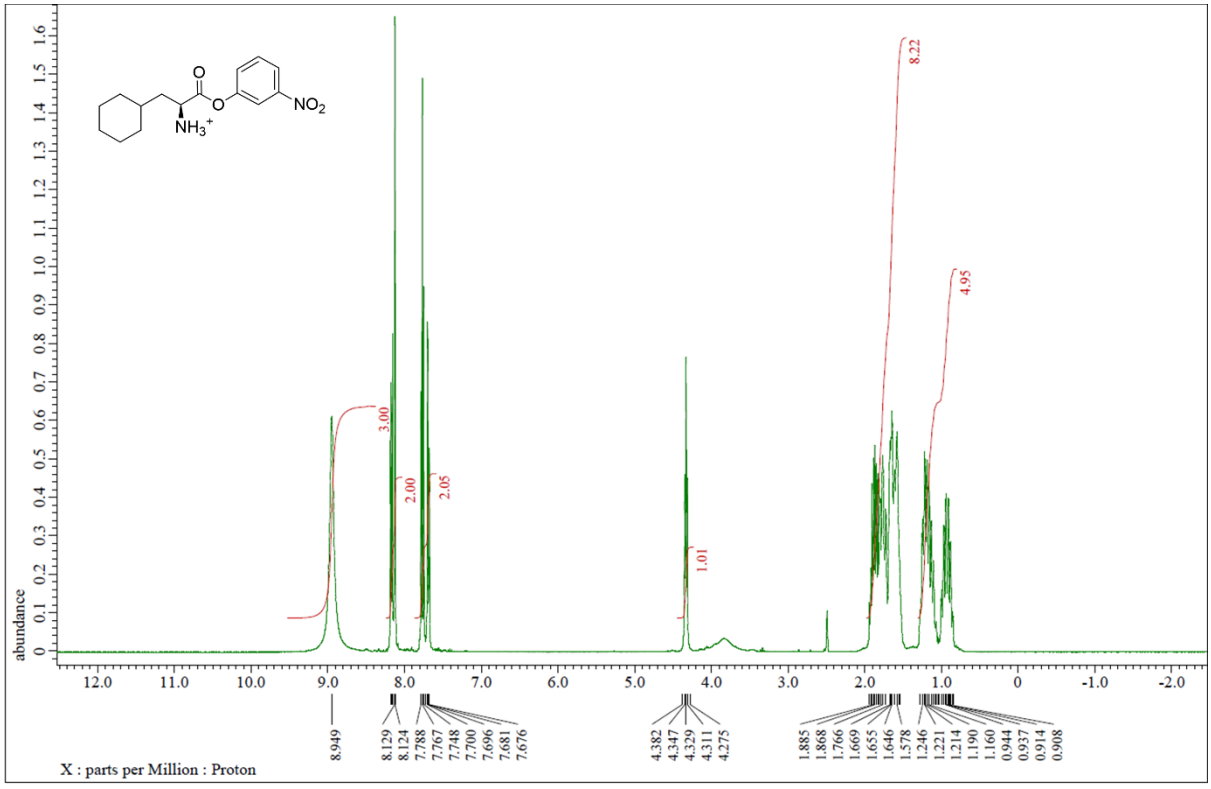


^{1}H NMR (400 MHz, $CDCl_3$) δ 8.13-8.10 (m, 1H), 8.00 (t, J = 2.1 Hz, 1H), 7.57 (t, J = 8.2 Hz, 1H), 7.48 (dd, J = 8.0, 1.1 Hz, 1H), 5.30-5.10 (m, 1H), 4.57-4.43 (m, 1H), 1.93-0.84 (m, 22H)

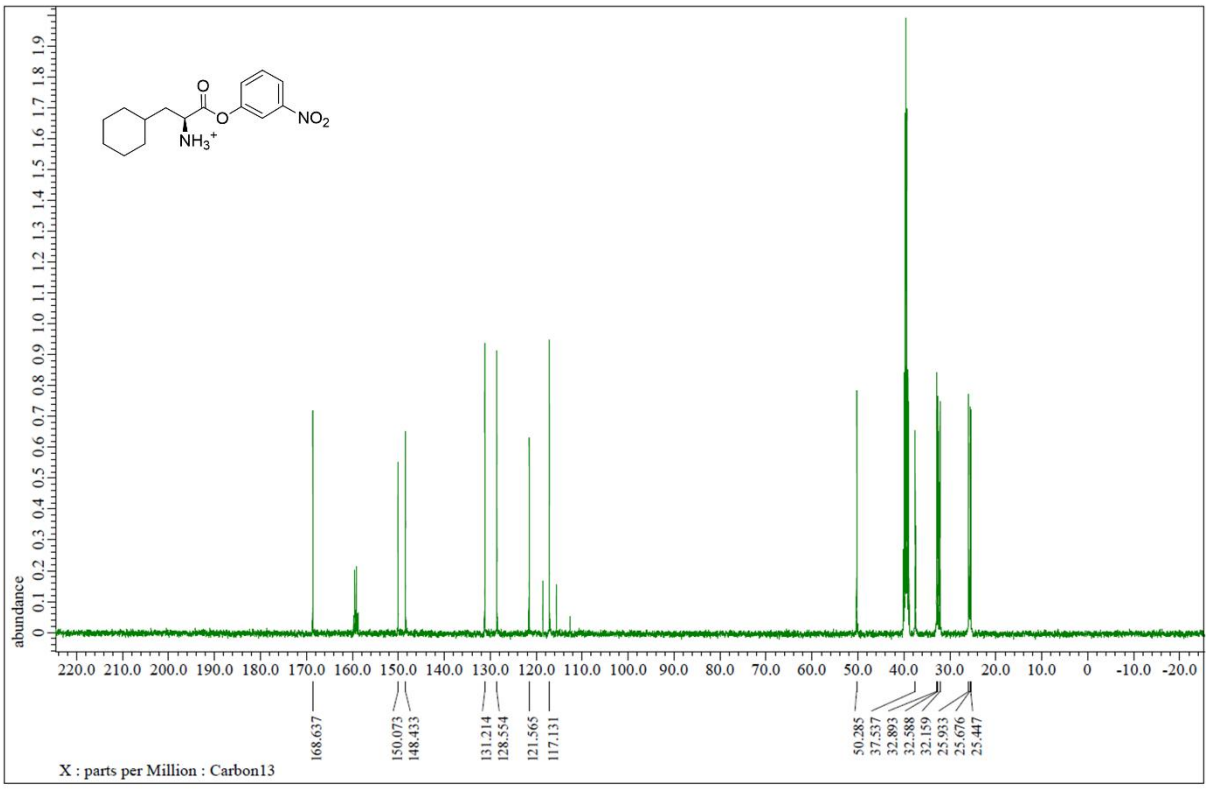


^{13}C NMR (101 MHz, $CDCl_3$) δ 171.7, 155.5, 150.8, 148.7, 130.0, 127.9, 120.8, 117.1, 80.2, 51.8, 39.6, 34.1, 33.5, 32.3, 28.2, 26.3, 26.1, 25.9

Supplementary Fig. 26. ^{1}H NMR and ^{13}C NMR spectra of Boc-Cha-3NP

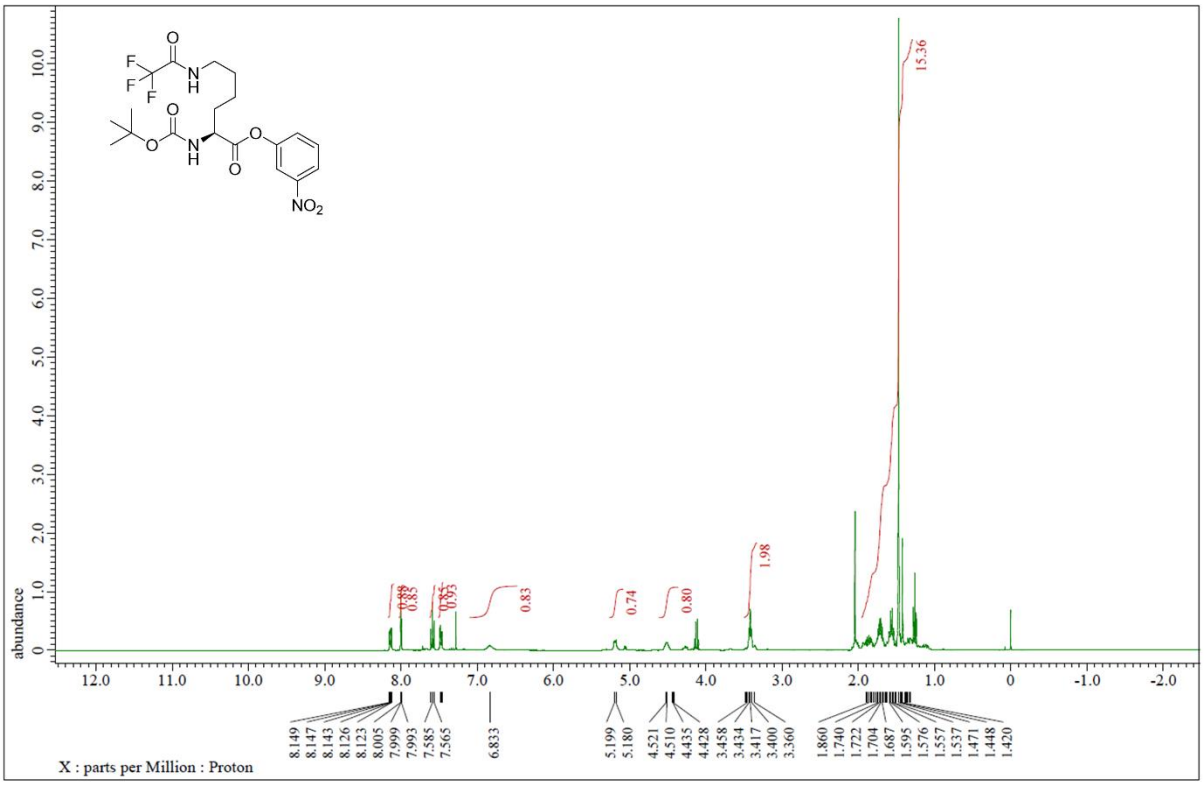


^{1}H NMR (400 MHz, DMSO-d_6) δ 8.95 (s, 3H), 8.18-8.12 (m, 2H), 7.79-7.68 (m, 2H), 4.38-4.27 (m, 1H), 1.94-1.54 (m, 8H), 1.28-0.85 (m, 5H)

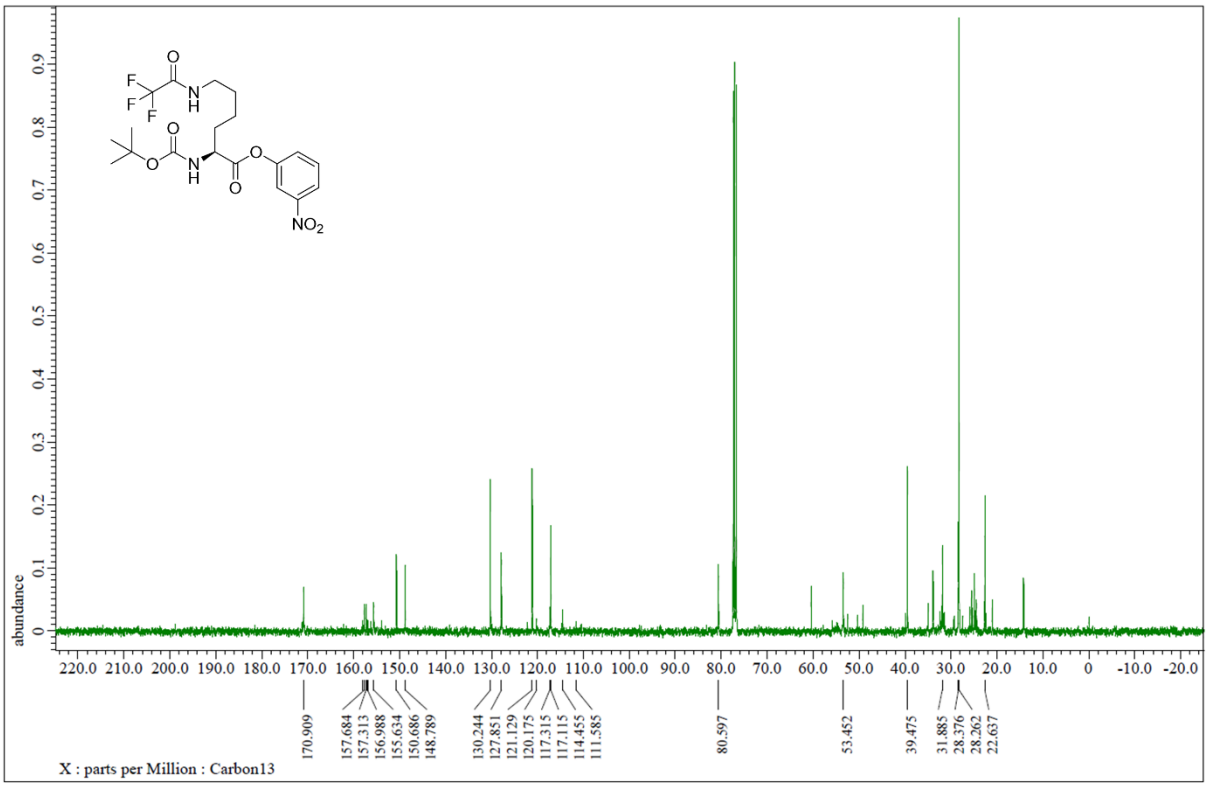


^{13}C NMR (101 MHz, DMSO-d_6) δ 168.6, 150.1, 148.4, 131.2, 128.6, 121.6, 117.1, 50.3, 37.5, 32.9, 32.6, 32.2, 25.9, 25.7, 25.4

Supplementary Fig. 27. ^{1}H NMR and ^{13}C NMR spectra of Cha-3NP

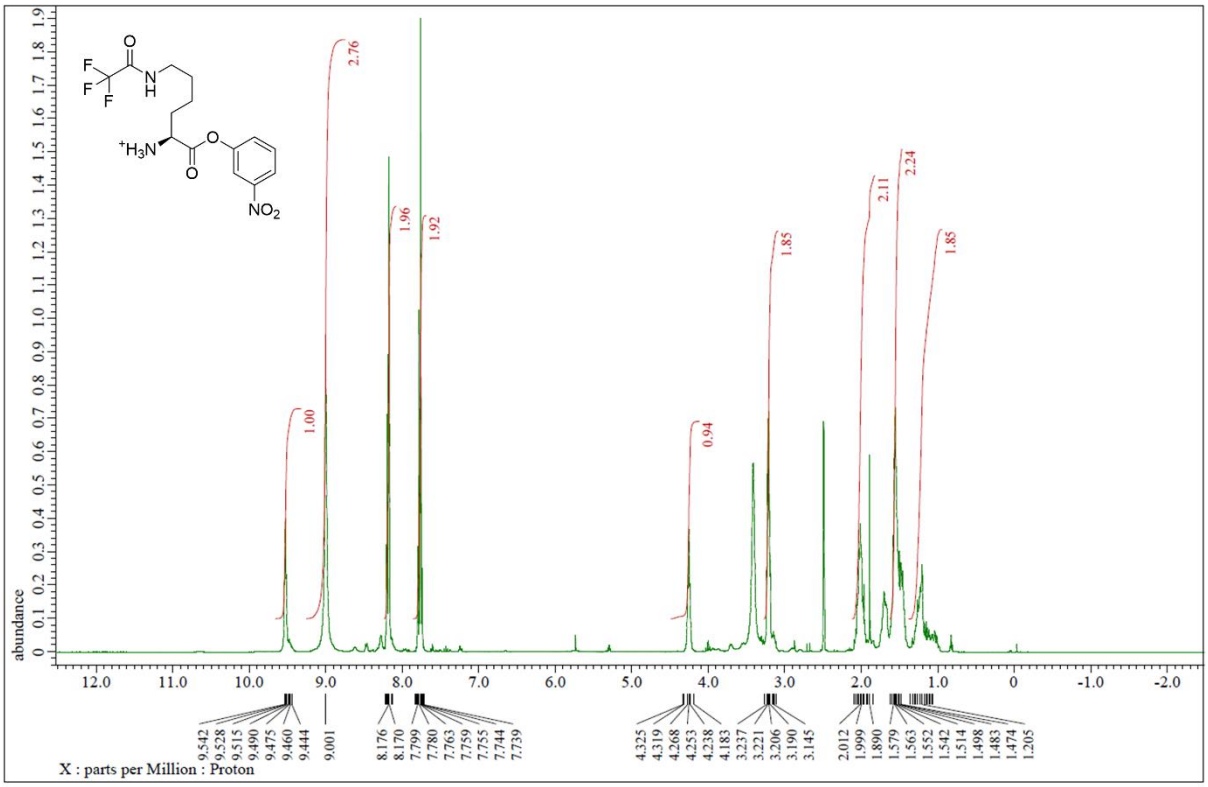


^{1}H NMR (400 MHz, $CDCl_3$) δ 8.15-8.12 (m, 1H), 8.00 (t, J = 2.3 Hz, 1H), 7.58 (t, J = 8.0 Hz, 1H), 7.49-7.46 (m, 1H), 6.83 (s, 1H), 5.19 (d, J = 7.8 Hz, 1H), 4.52-4.42 (m, 1H), 3.48-3.36 (m, 2H), 1.90-1.32 (m, 15H)

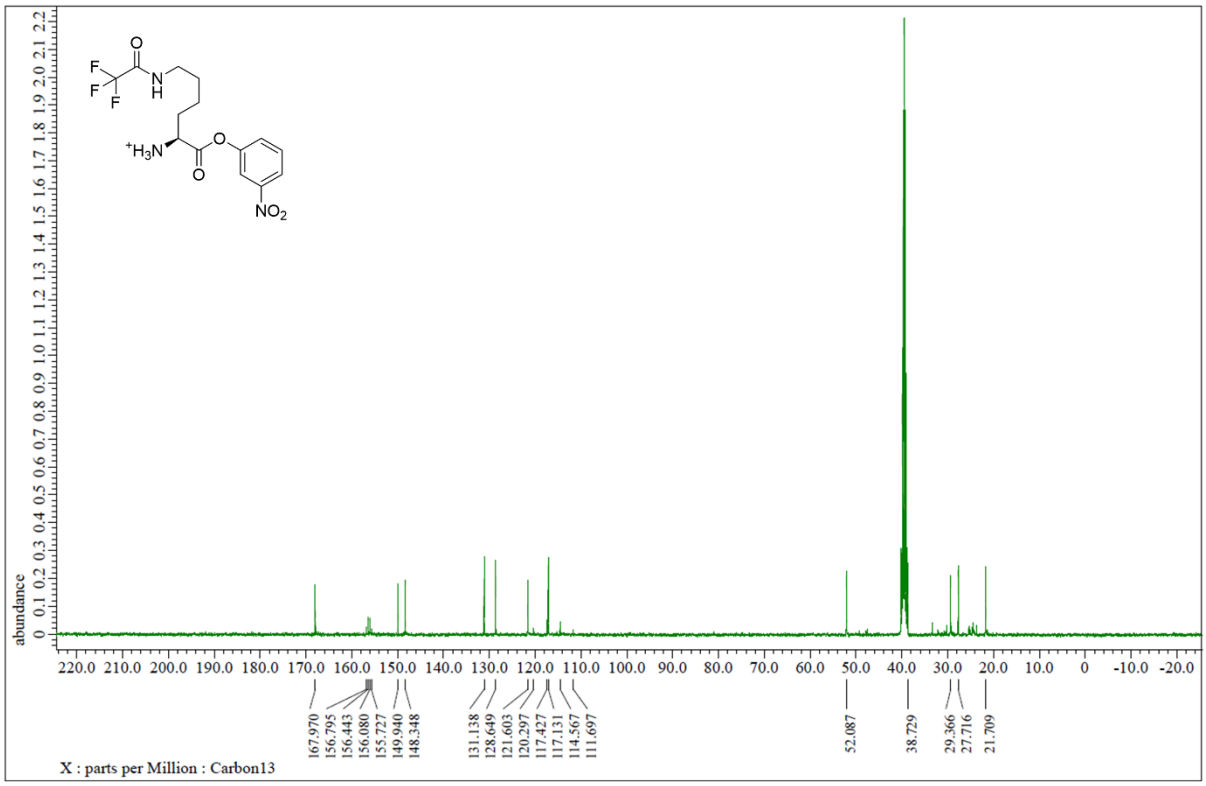


^{13}C NMR (101 MHz, $CDCl_3$) δ 170.9, 157.5 (q, J = 36.7 Hz), 155.6, 150.7, 148.8, 130.2, 127.9, 121.1, 117.1, 115.9 (q, J = 287.9 Hz), 80.6, 53.5, 39.5, 31.9, 28.4, 28.3, 22.6

Supplementary Fig. 28. ^{1}H NMR and ^{13}C NMR spectra of Boc-L-Lys(Tfa)-3NP

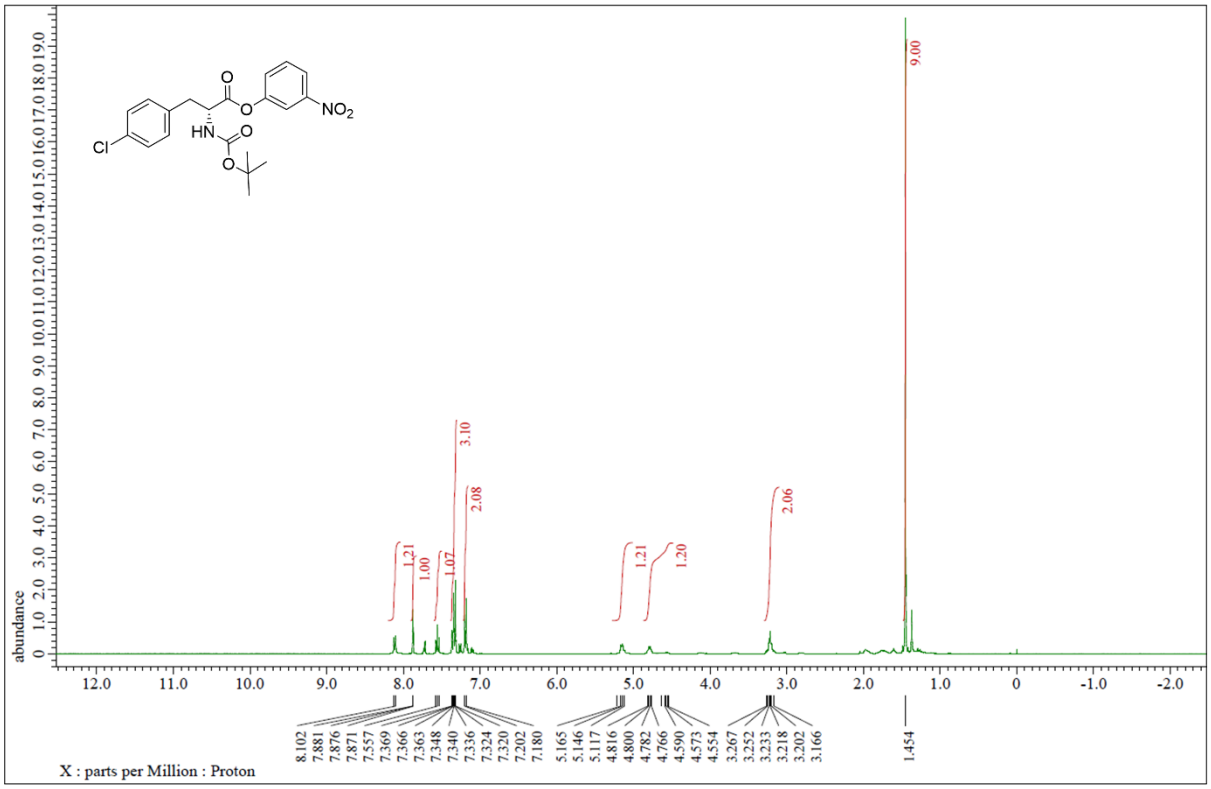


^{1}H NMR (400 MHz, DMSO-d_6) δ 9.54-9.44 (m, 1H), 9.00 (s, 3H), 8.22-8.13 (m, 2H), 7.84-7.72 (m, 2H), 4.33-4.18 (m, 1H), 3.26-3.12 (m, 2H), 2.09-1.85 (m, 2H), 1.62-1.47 (m, 2H), 1.36-1.06 (m, 2H)

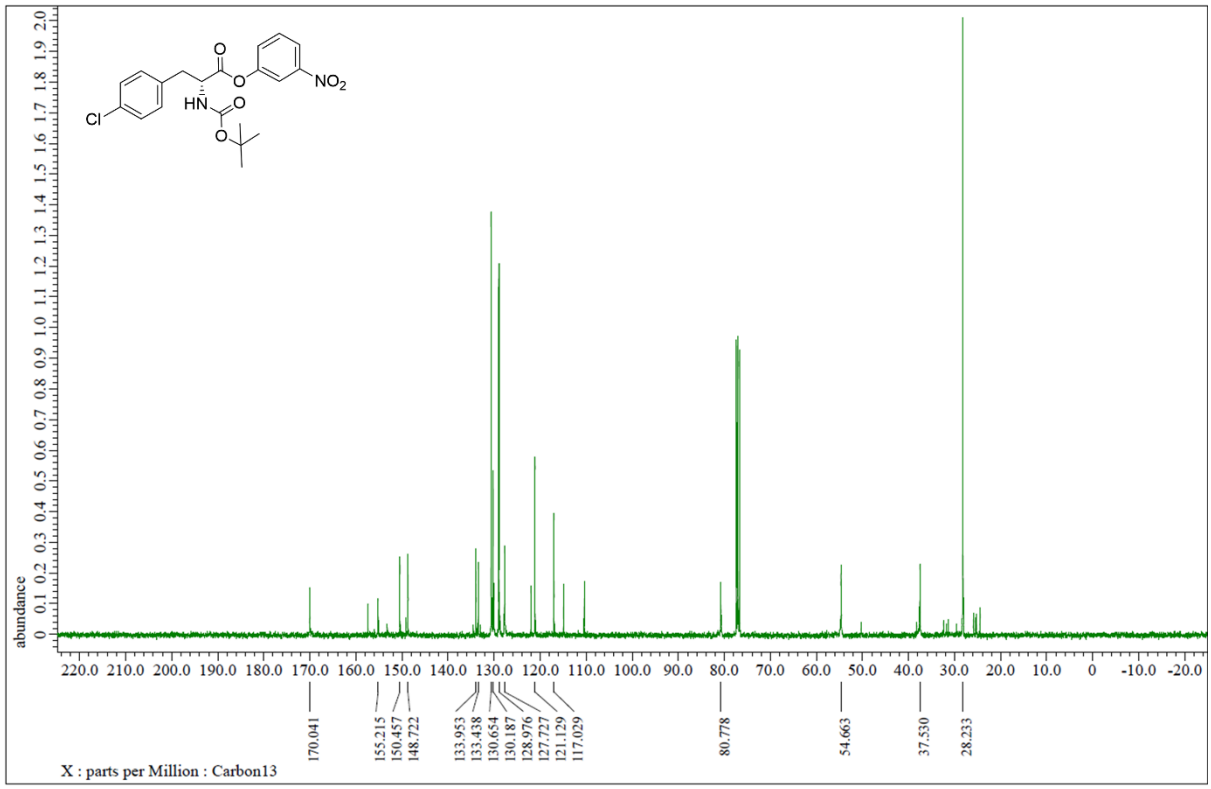


^{13}C NMR (101 MHz, DMSO-d_6) δ 168.0, 156.3 (q, J = 35.8 Hz), 149.9, 148.3, 131.1, 128.6, 121.6, 117.1, 116.0 (q, J = 288.2 Hz), 52.1, 38.7, 29.4, 27.7, 21.7

Supplementary Fig. 29. ^{1}H NMR and ^{13}C NMR spectra of L-Lys(Tfa)-3NP

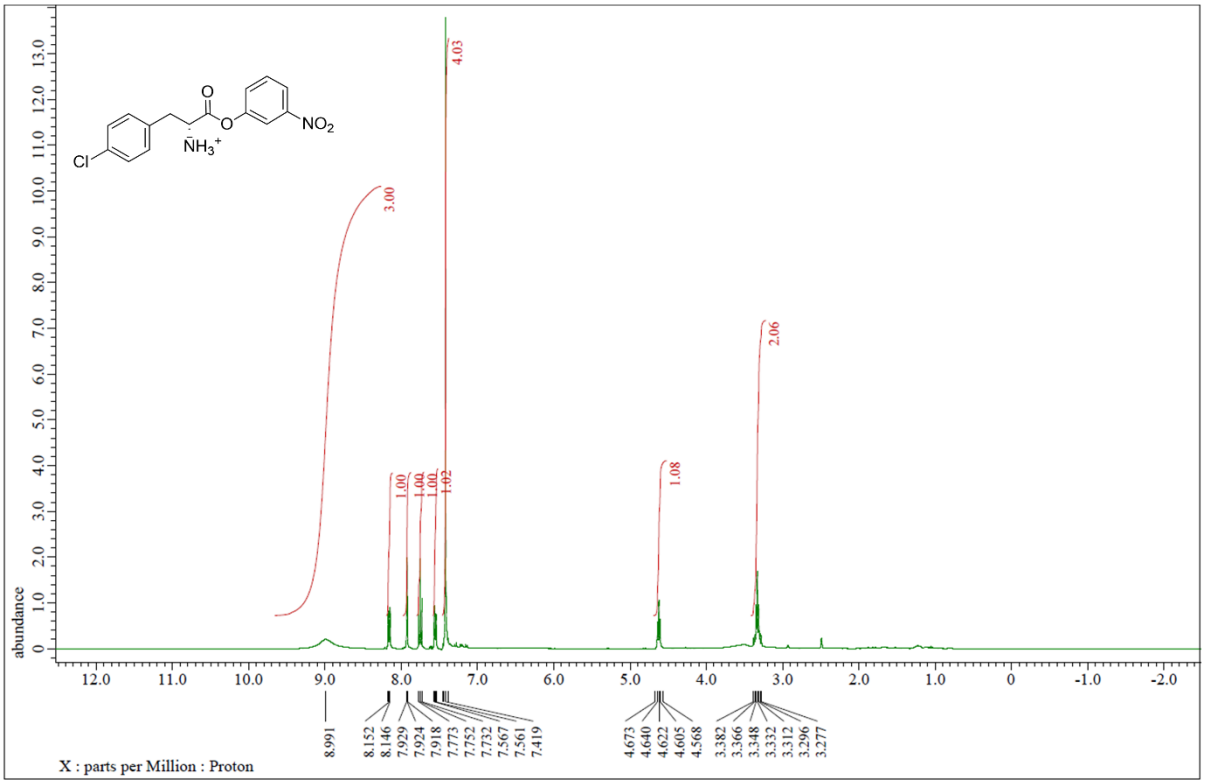


^{1}H NMR (400 MHz, $CDCl_3$) δ 8.11 (dd, J = 8.2, 0.9 Hz, 1H), 7.88 (t, J = 2.1 Hz, 1H), 7.56 (t, J = 8.2 Hz, 1H), 7.37-7.32 (m, 3H), 7.19 (d, J = 8.7 Hz, 2H), 5.21-5.12 (m, 1H), 4.82-4.54 (m, 1H), 3.27-3.17 (m, 2H), 1.45 (s, 9H)

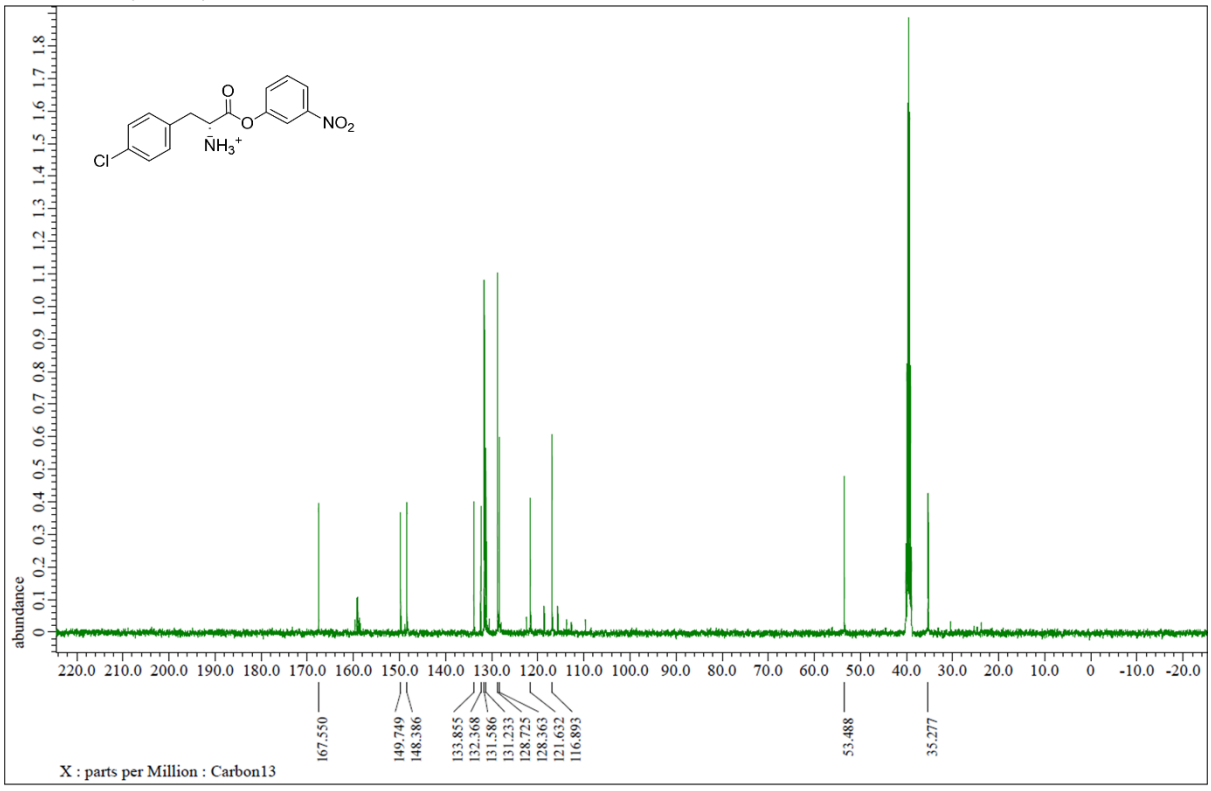


^{13}C NMR (101 MHz, $CDCl_3$) δ 170.0, 155.2, 150.5, 148.7, 134.0, 133.4, 130.7, 130.2, 129.0, 127.7, 121.1, 117.0, 80.8, 54.7, 37.5, 28.2

Supplementary Fig. 30. ^{1}H NMR and ^{13}C NMR spectra of Boc-D-Phe(4-Cl)-3NP

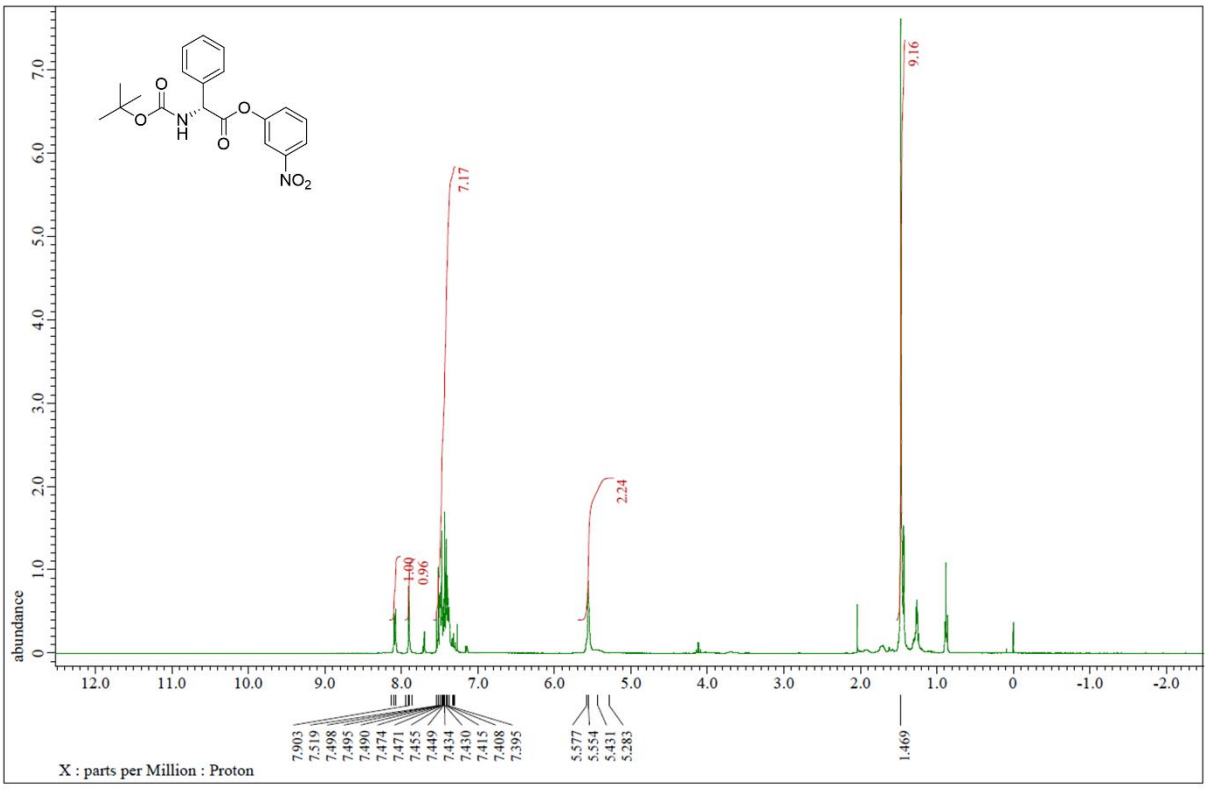


^{1}H NMR (400 MHz, DMSO-d_6) δ 8.99 (s, 3H), 8.16 (dd, J = 8.2, 2.3 Hz, 1H), 7.92 (t, J = 2.1 Hz, 1H), 7.75 (t, J = 8.2 Hz, 1H), 7.55 (dd, J = 8.0, 2.1 Hz, 1H), 7.46-7.38 (m, 4H), 4.67-4.57 (m, 1H), 3.38-3.28 (m, 2H)

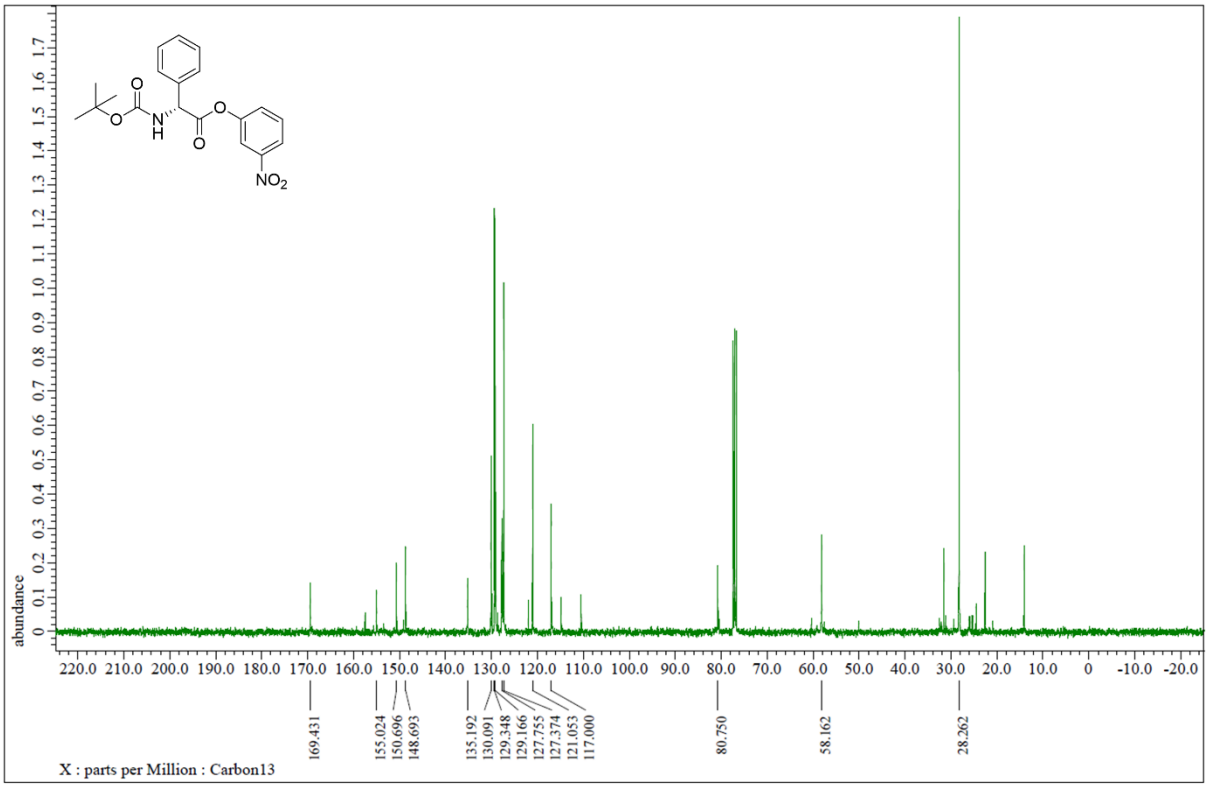


^{13}C NMR (101 MHz, DMSO-d_6) δ 167.6, 149.7, 148.4, 133.9, 132.4, 131.6, 131.2, 128.7, 128.4, 121.6, 116.9, 53.5, 35.3

Supplementary Fig. 31. ^{1}H NMR and ^{13}C NMR spectra of D-Phe(4-Cl)-3NP

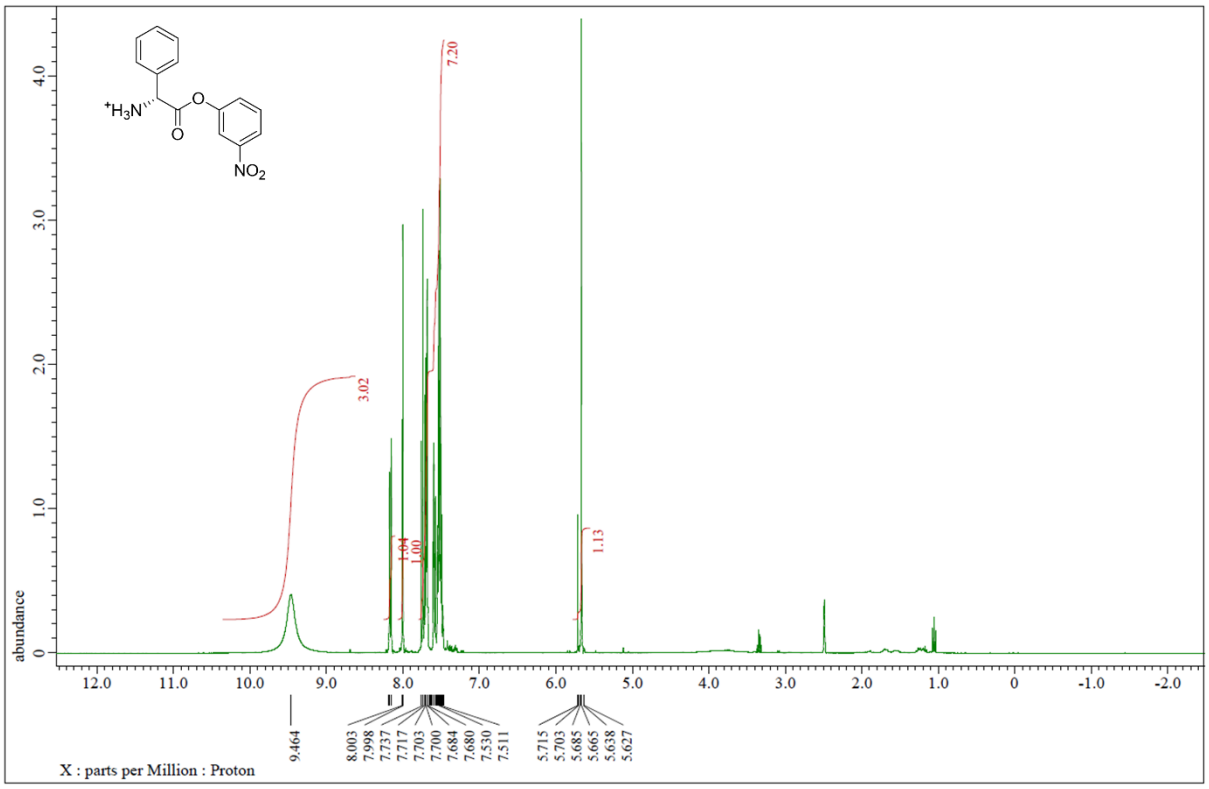


^{1}H NMR (400 MHz, $CDCl_3$) δ 8.13-8.07 (m, 1H), 7.94-7.86 (m, 1H), 7.54-7.30 (m, 7H), 5.58-5.28 (m, 2H), 1.47 (s, 9H)

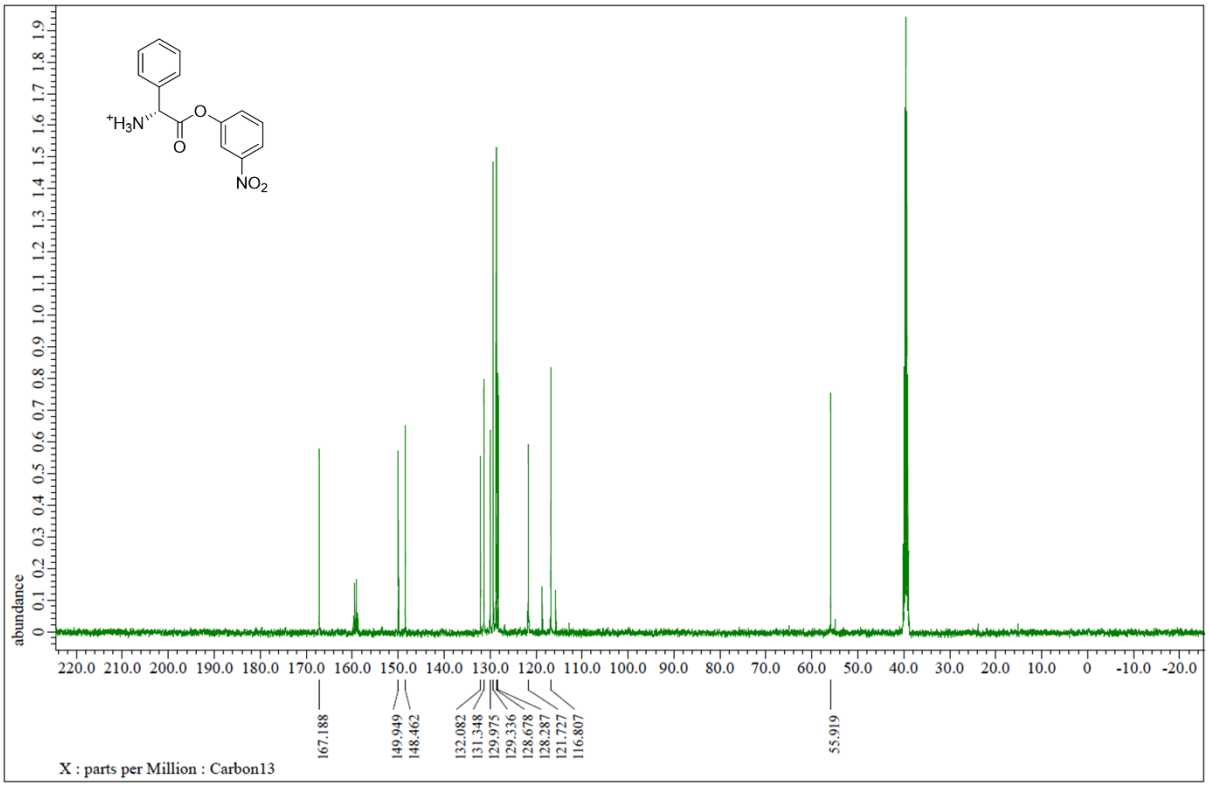


^{13}C NMR (101 MHz, $CDCl_3$) δ 169.4, 155.0, 150.7, 148.7, 135.2, 130.1, 129.3, 129.2, 127.8, 127.4, 121.1, 117.0, 80.7, 58.2, 28.3

Supplementary Fig. 32. ^{1}H NMR and ^{13}C NMR spectra of Boc-D-Phg-3NP

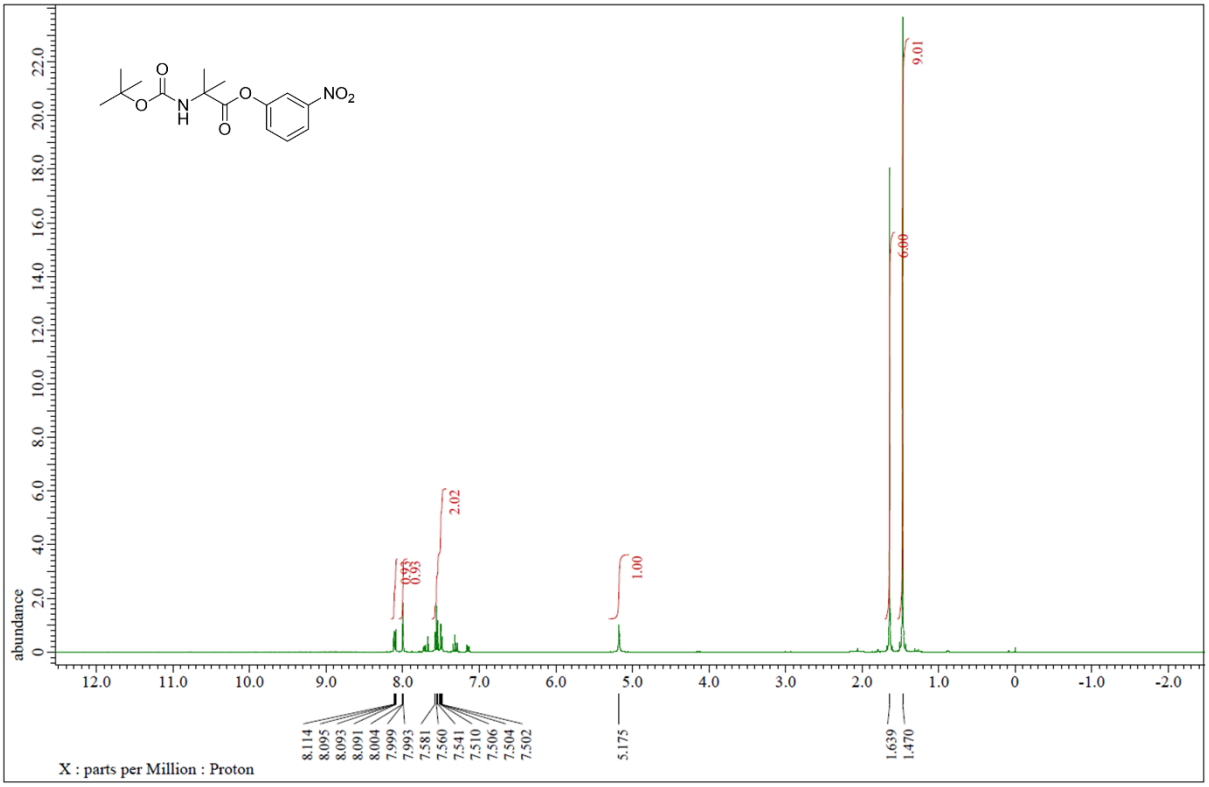


^{1}H NMR (400 MHz, DMSO-d_6) δ 9.46 (s, 3H), 8.19-8.15 (m, 1H), 8.00 (t, J = 2.1 Hz, 1H), 7.76-7.47 (m, 7H), 5.71-5.63 (m, 1H)

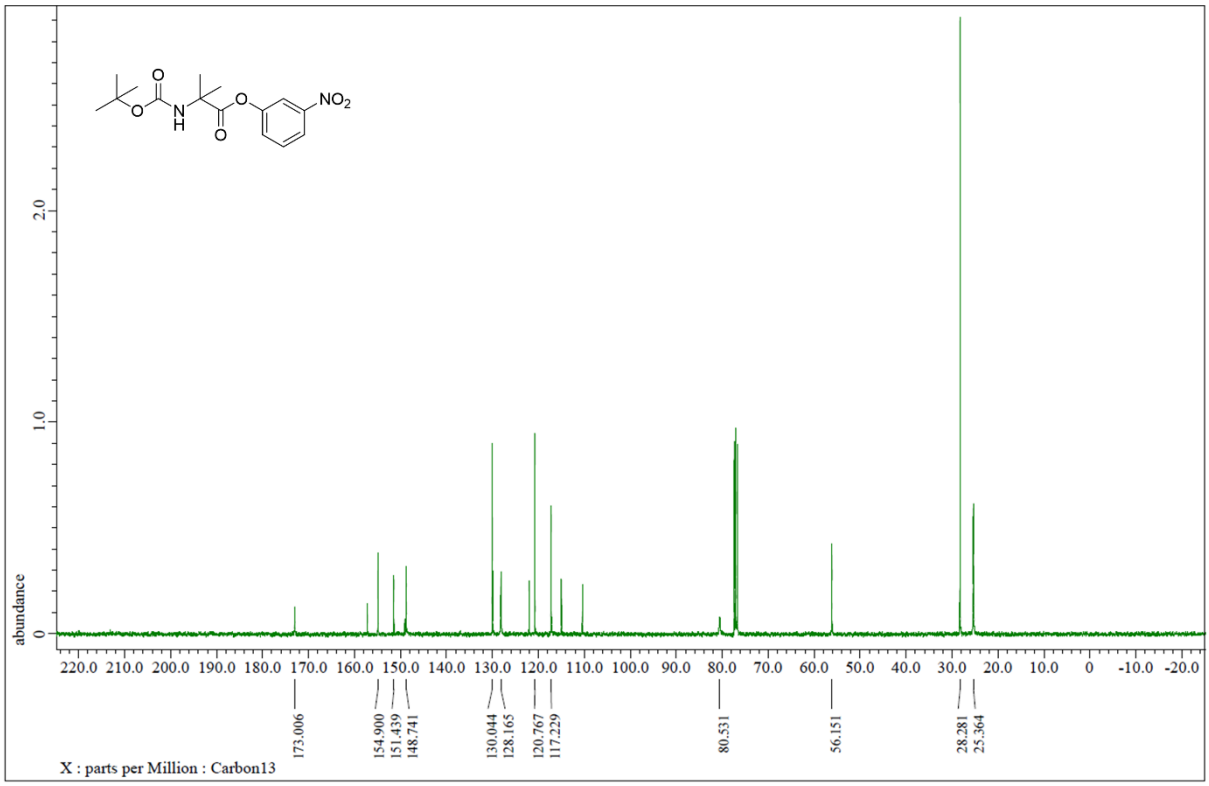


^{13}C NMR (101 MHz, DMSO-d_6) δ 167.2, 149.9, 148.5, 132.1, 131.3, 130.0, 129.3, 128.7, 128.3, 121.7, 116.8, 55.9

Supplementary Fig. 33. ^{1}H NMR and ^{13}C NMR spectra of D-Phg-3NP

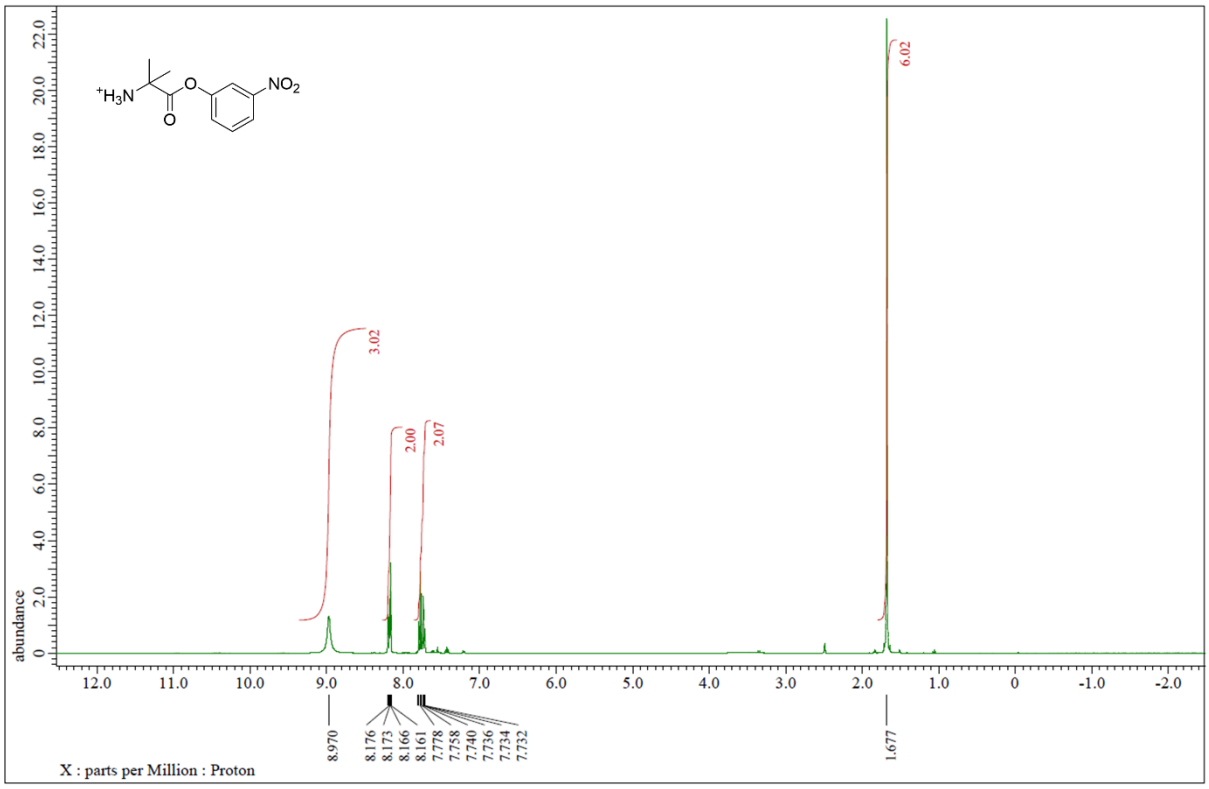


^{1}H NMR (400 MHz, $CDCl_3$) δ 8.12-8.09 (m, 1H), 8.00 (t, J = 2.1 Hz, 1H), 7.58-7.48 (m, 2H), 5.18 (s, 1H), 1.64 (s, 6H), 1.47 (s, 9H)

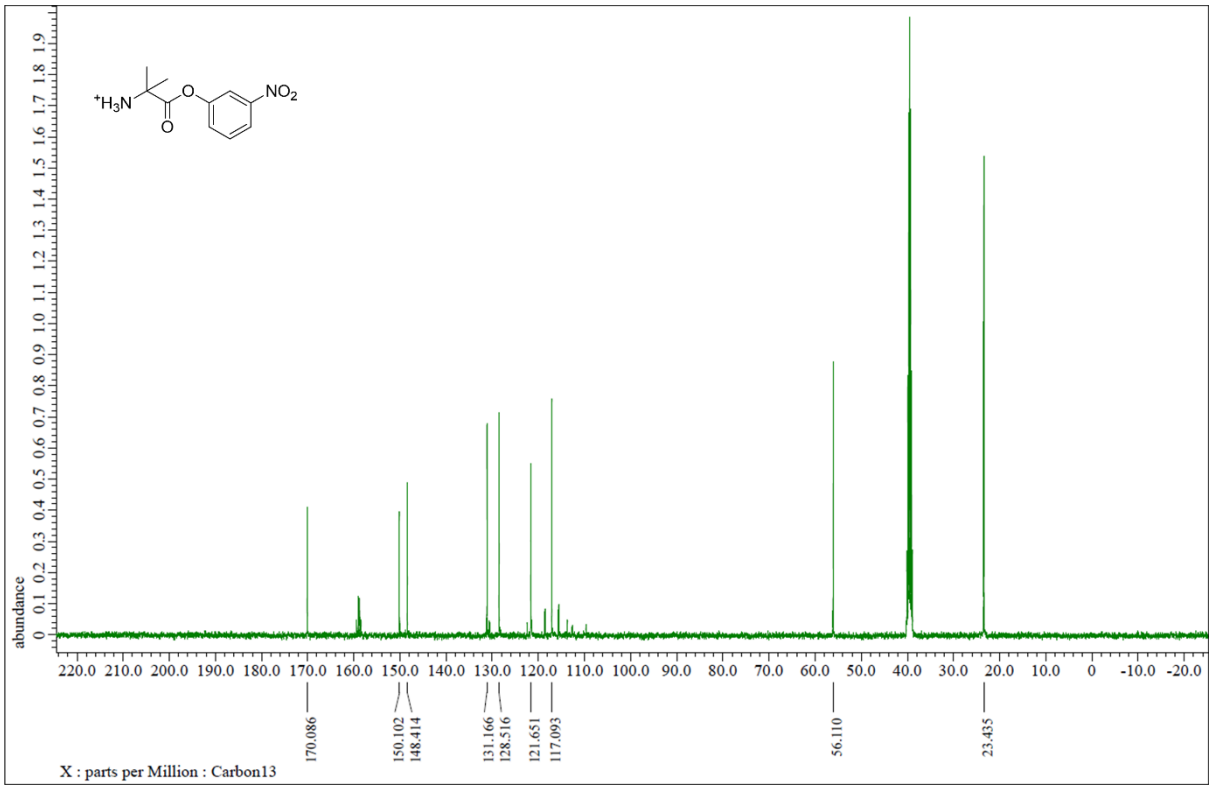


^{13}C NMR (101 MHz, $CDCl_3$) δ 173.0, 154.9, 151.4, 148.7, 130.0, 128.2, 120.8, 117.2, 80.5, 56.2, 28.3, 25.4

Supplementary Fig. 34. ^{1}H NMR and ^{13}C NMR spectra of Boc-Aib-3NP

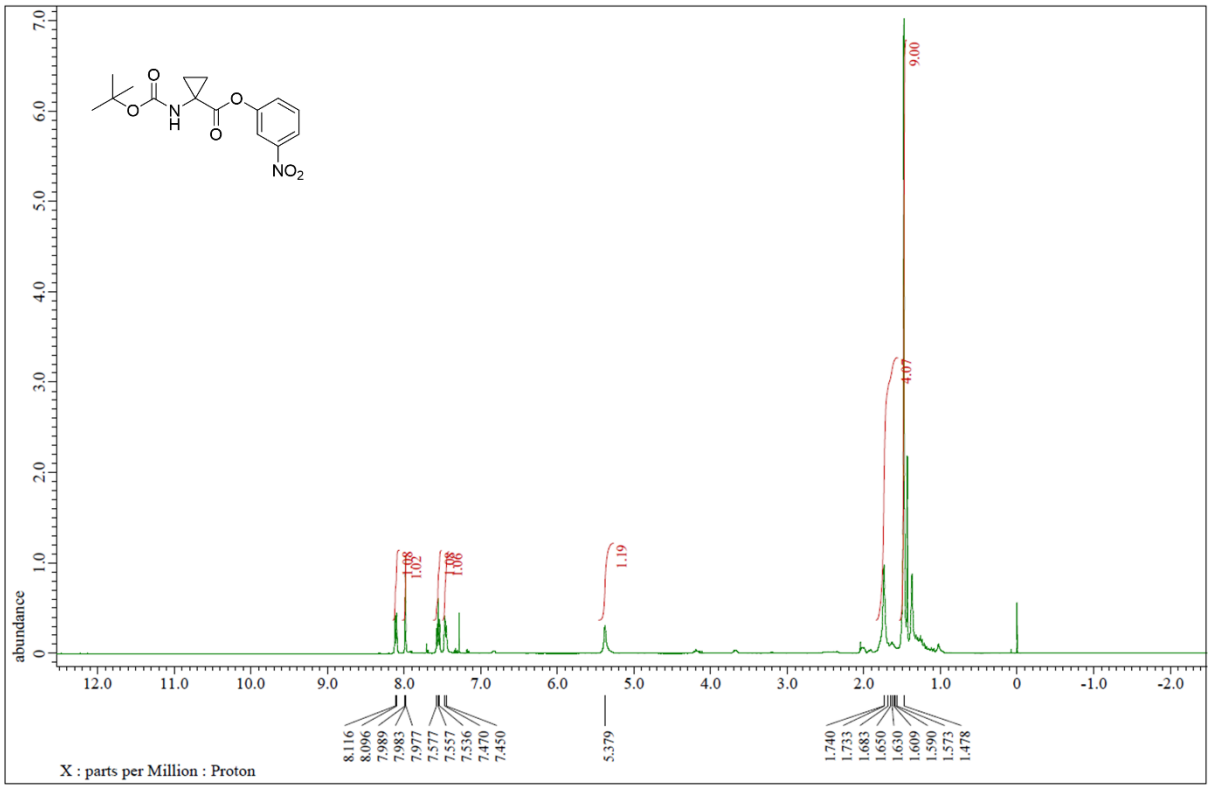


^{1}H NMR (400 MHz, DMSO-d_6) δ 8.97 (s, 3H), 8.20-8.16 (m, 2H), 7.81-7.71 (m, 2H), 1.68 (s, 6H)

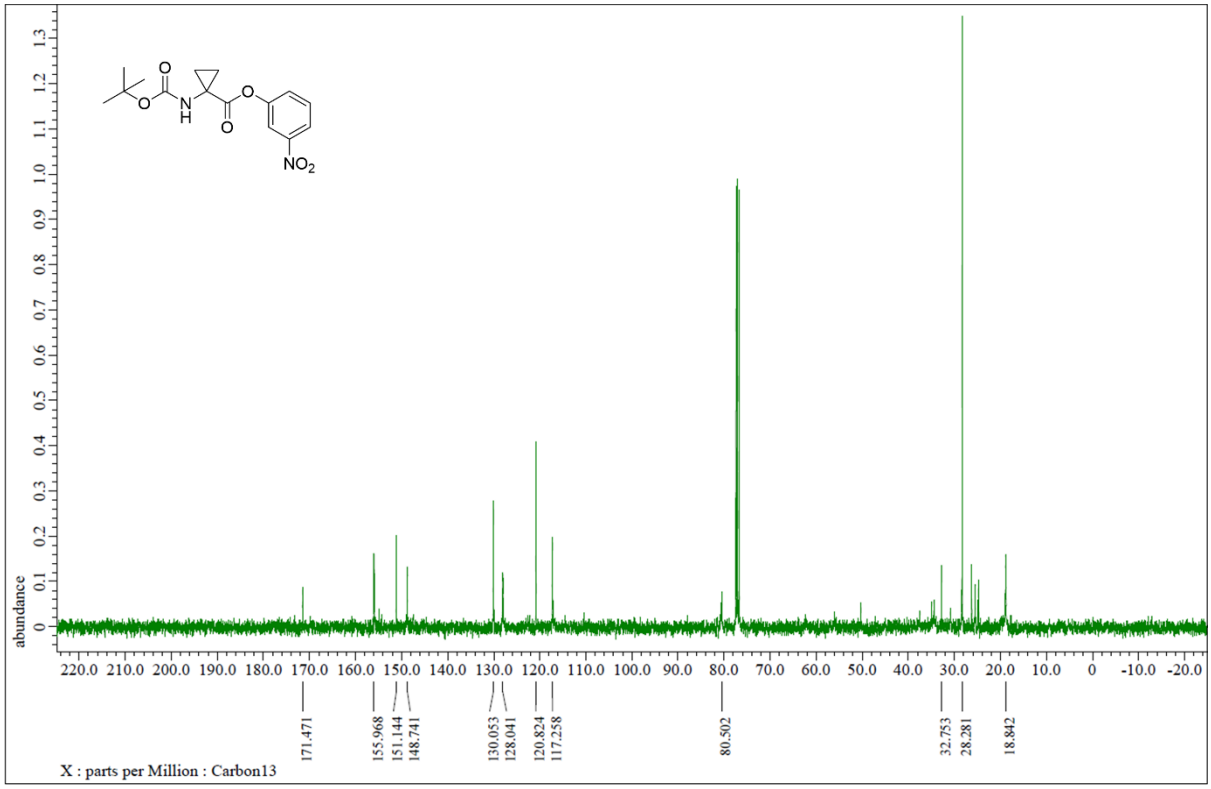


^{13}C NMR (101 MHz, DMSO-d_6) δ 170.1, 150.1, 148.4, 131.2, 128.5, 121.7, 117.1, 56.1, 23.4

Supplementary Fig. 35. ^{1}H NMR and ^{13}C NMR spectra of Aib-3NP

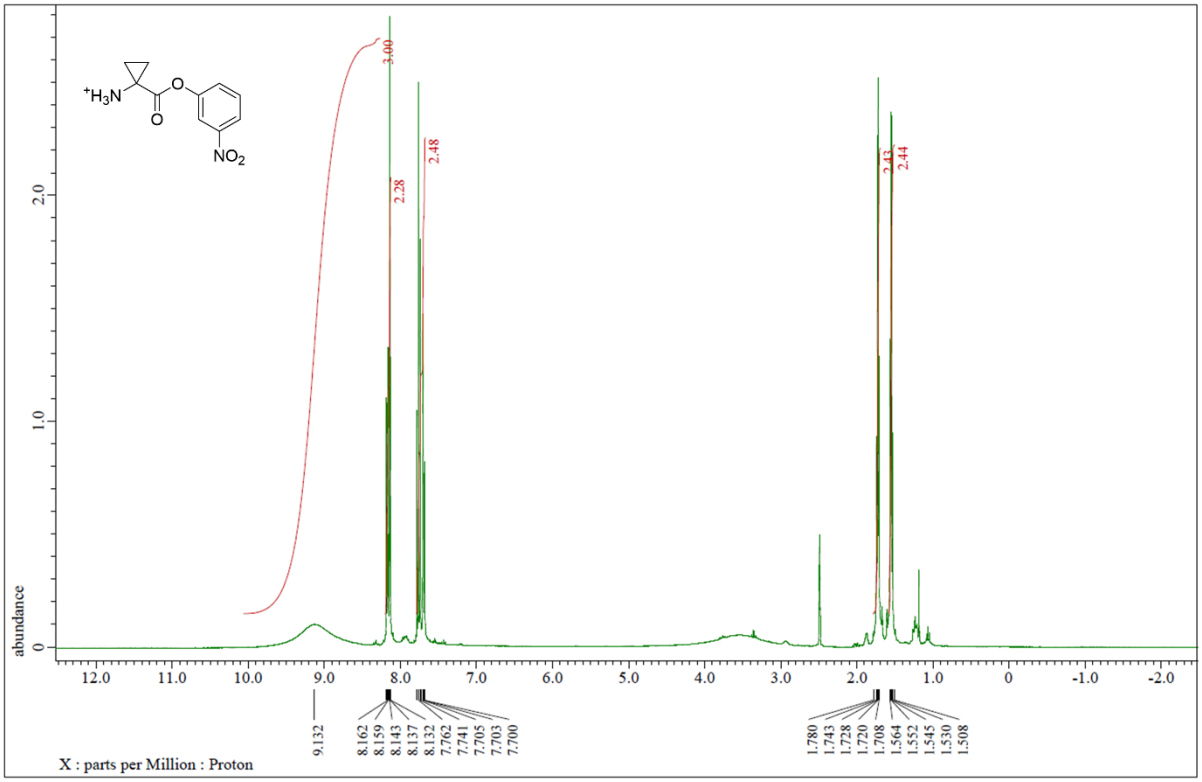


^{1}H NMR (400 MHz, $CDCl_3$) δ 8.11 (d, J = 7.8 Hz, 1H), 7.98 (t, J = 2.3 Hz, 1H), 7.56 (t, J = 8.2 Hz, 1H), 7.46 (d, J = 7.8 Hz, 1H), 5.38 (s, 1H), 1.74-1.57 (m, 4H), 1.48 (s, 9H)

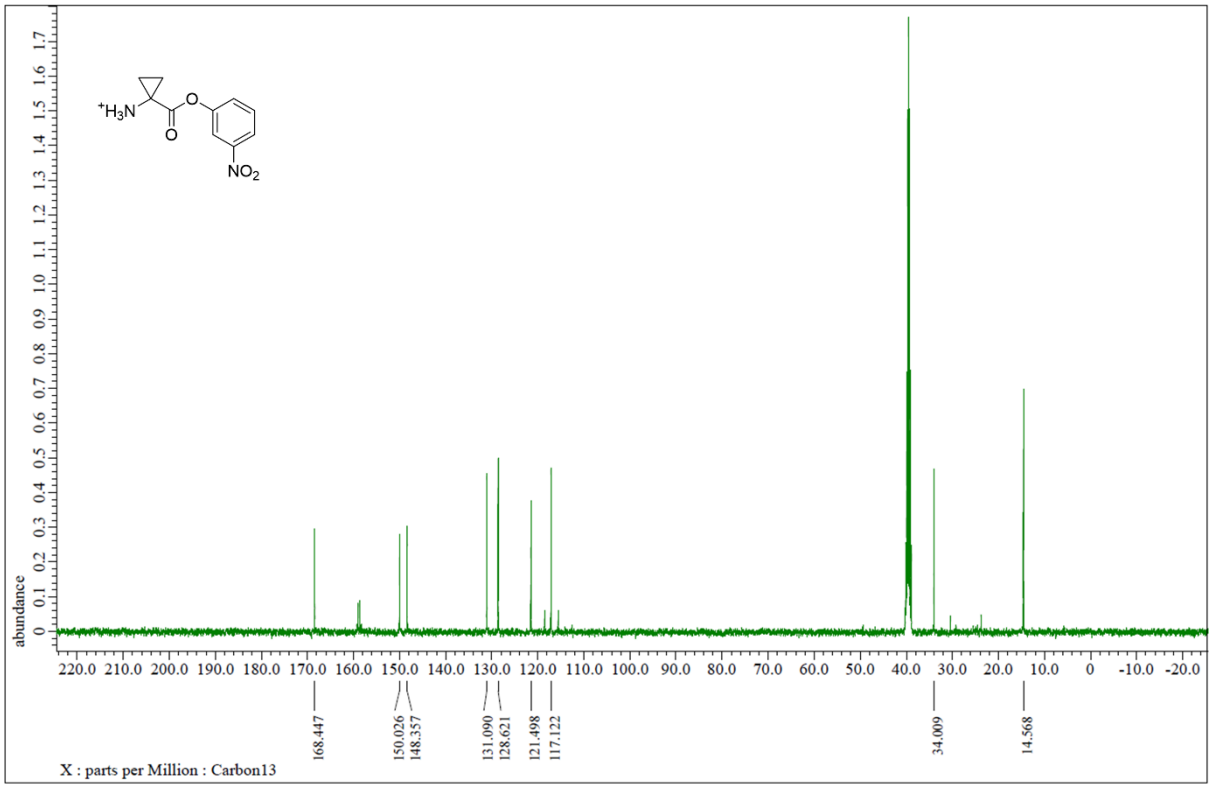


^{13}C NMR (101 MHz, $CDCl_3$) δ 171.5, 156.0, 151.1, 148.7, 130.1, 128.0, 120.8, 117.3, 80.5, 32.8, 28.3, 18.8

Supplementary Fig. 36. ^{1}H NMR and ^{13}C NMR spectra of Boc-Acp-3NP

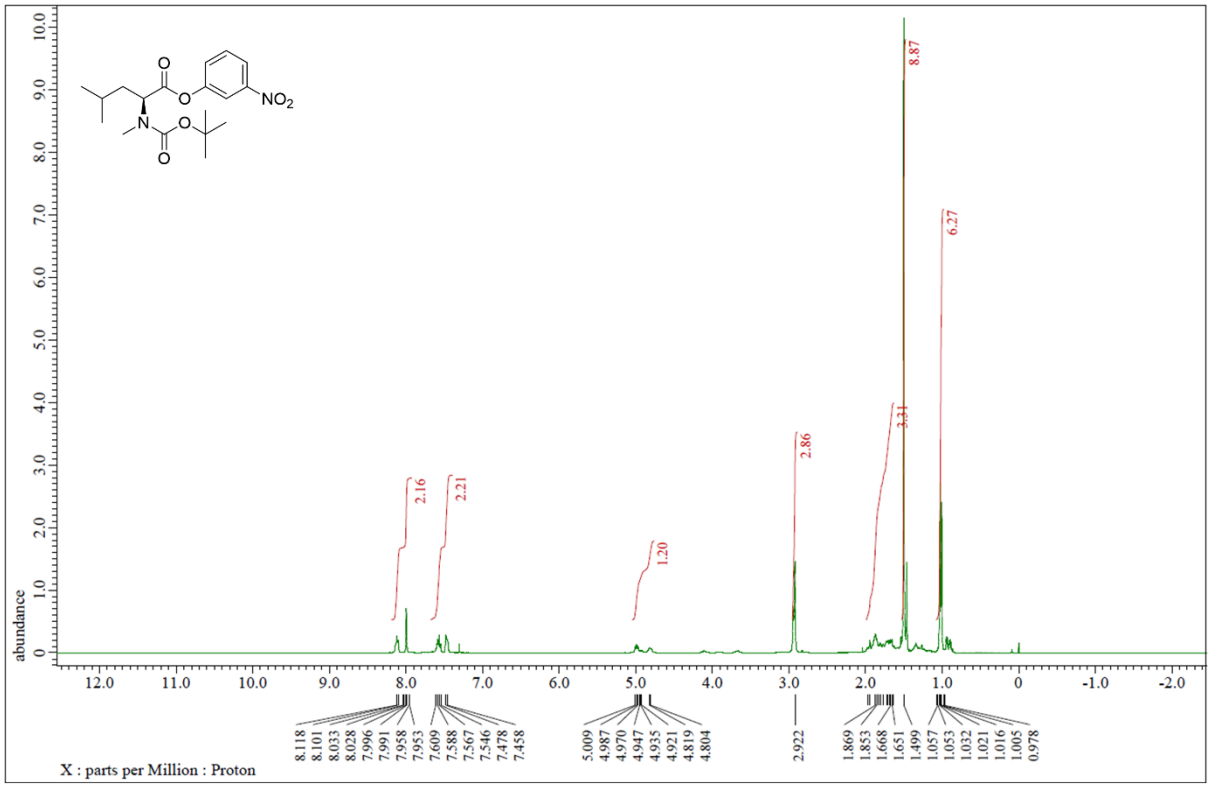


^{1}H NMR (400 MHz, DMSO-d_6) δ 9.13 (s, 3H), 8.19-8.13 (m, 2H), 7.78-7.68 (m, 2H), 1.78-1.71 (m, 2H), 1.56-1.51 (m, 2H)

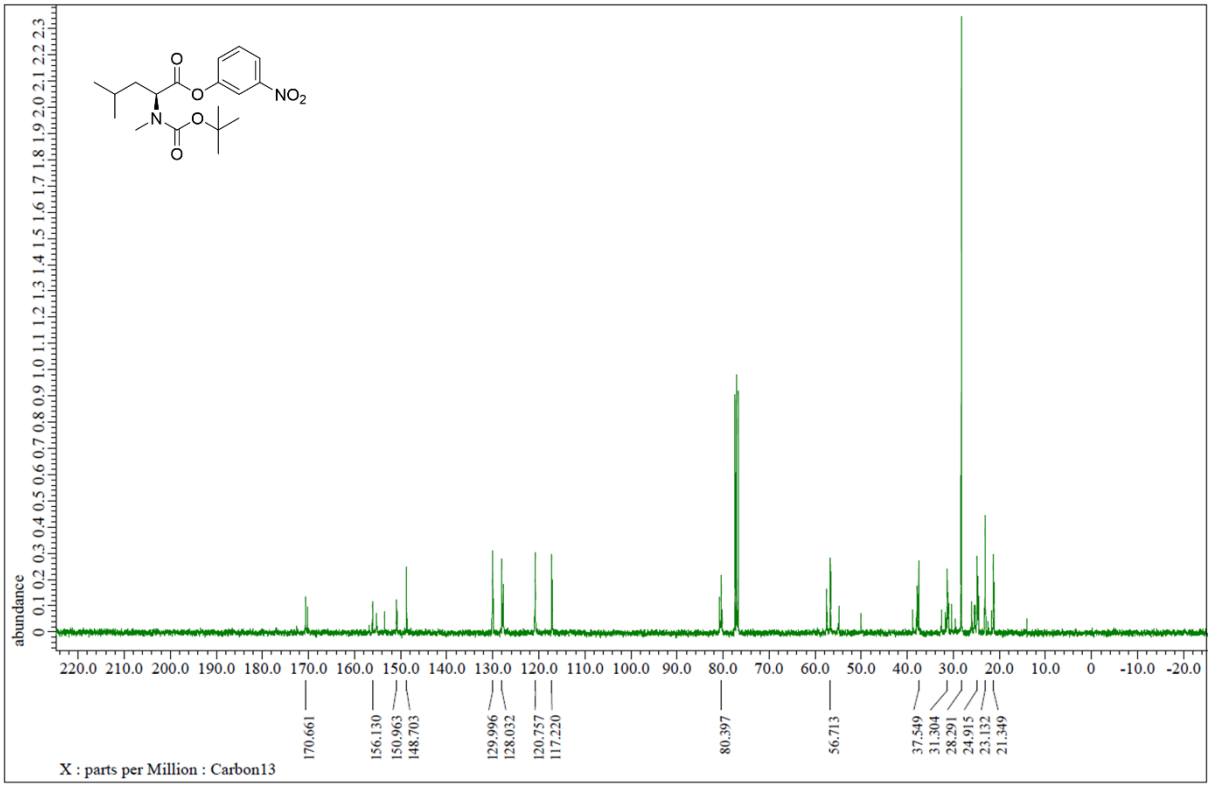


^{13}C NMR (101 MHz, DMSO-d_6) δ 168.4, 150.0, 148.4, 131.1, 128.6, 121.5, 117.1, 34.0, 14.6

Supplementary Fig. 37. ^{1}H NMR and ^{13}C NMR spectra of Acp-3NP

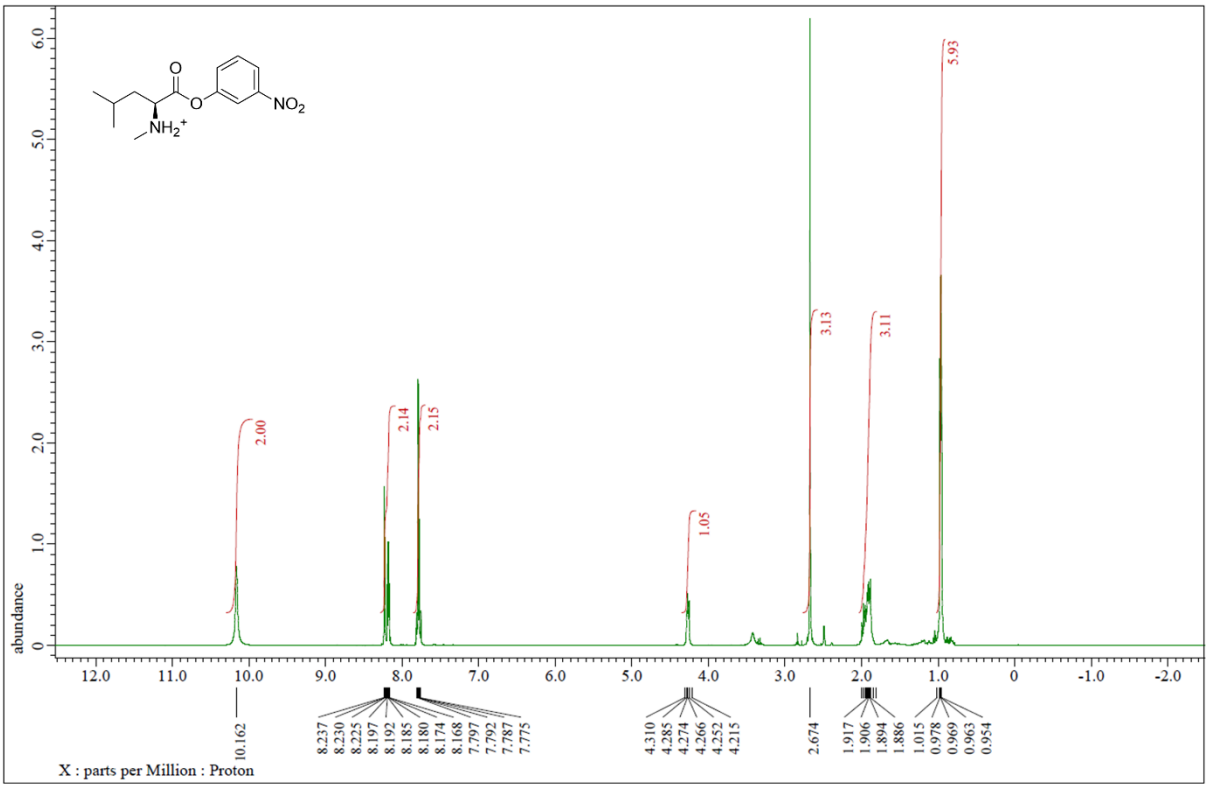


^{1}H NMR (400 MHz, $CDCl_3$) δ 8.12-7.95 (m, 2H), 7.61-7.46 (m, 2H), 5.01-4.80 (m, 1H), 2.92 (s, 3H), 1.97-1.63 (m, 3H), 1.50 (s, 9H), 1.07-1.00 (m, 6H)

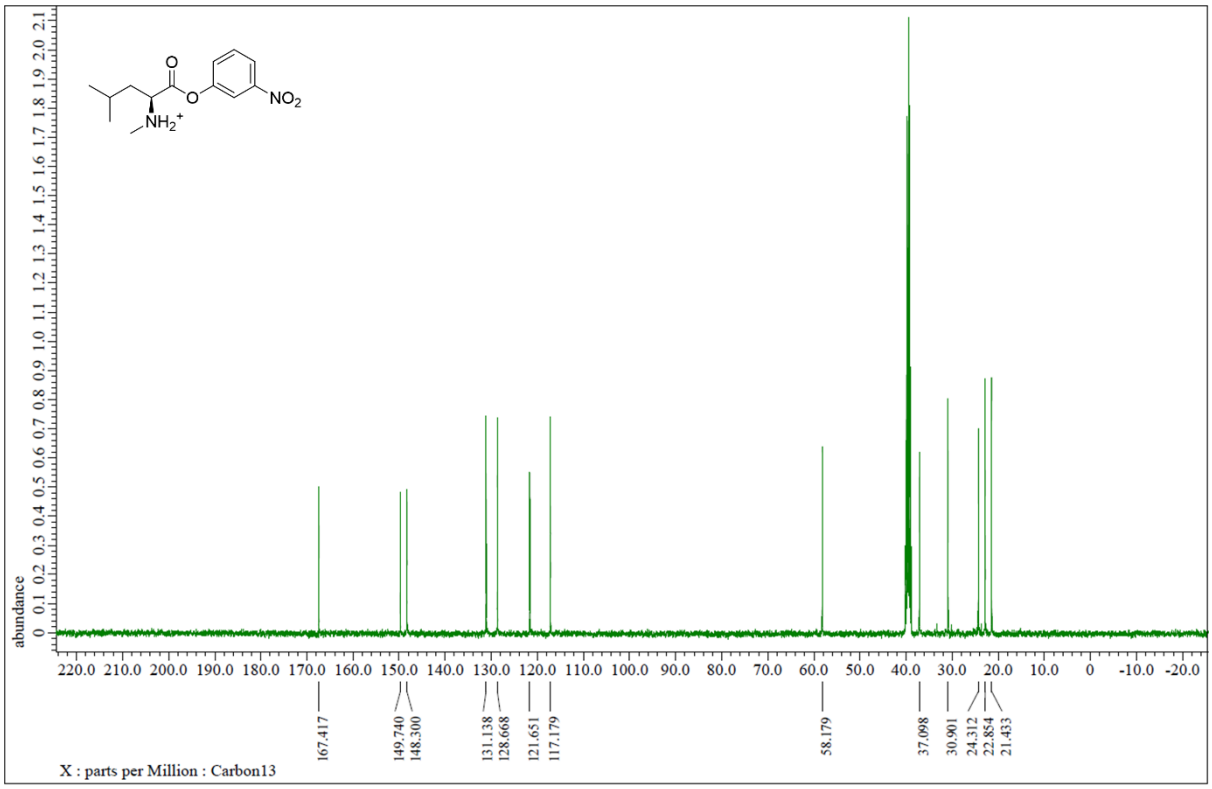


^{13}C NMR (101 MHz, $CDCl_3$) δ 170.7, 156.1, 151.0, 148.7, 130.0, 128.0, 120.8, 117.2, 80.4, 56.7, 37.5, 31.3, 28.3, 24.9, 23.1, 21.3

Supplementary Fig. 38. ^{1}H NMR and ^{13}C NMR spectra of Boc-Me-L-Leu-3NP

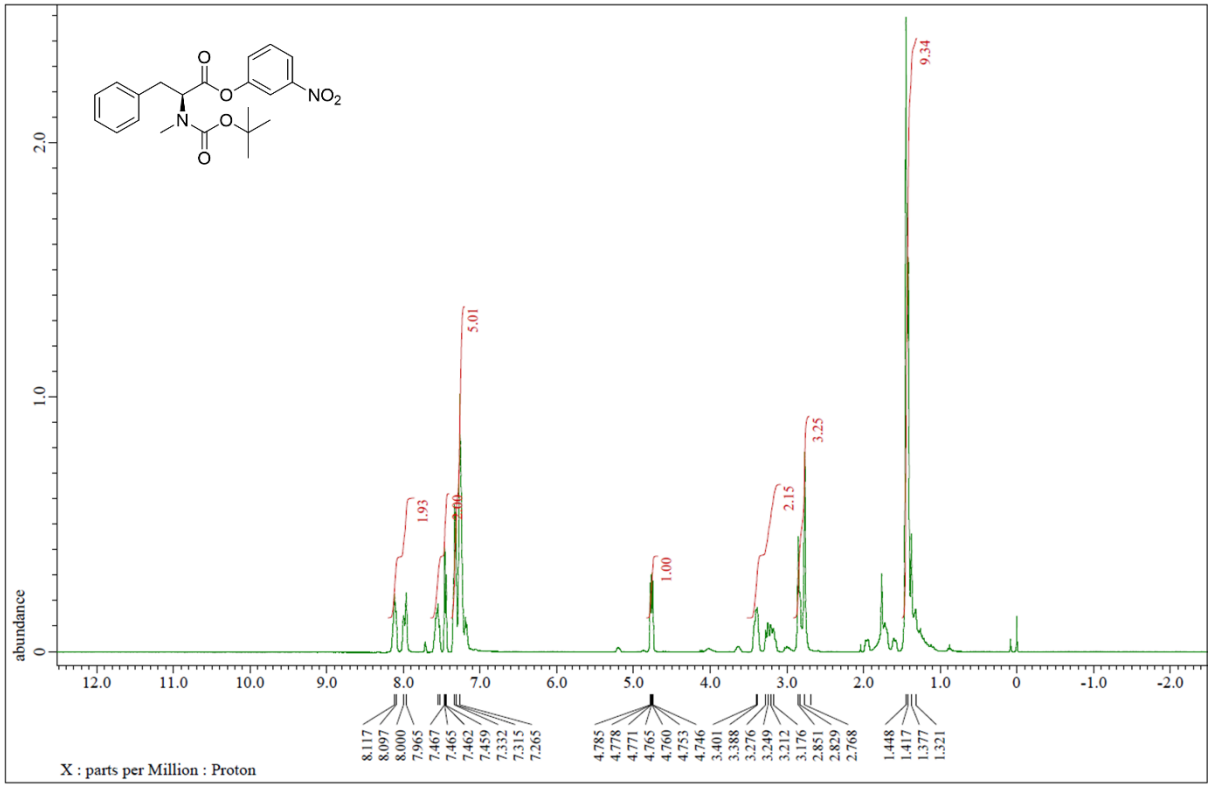


^{1}H NMR (400 MHz, DMSO-d_6) δ 10.16 (s, 2H), 8.24-8.17 (m, 2H), 7.81-7.75 (m, 2H), 4.31-4.22 (m, 1H), 2.67 (s, 3H), 1.99-1.81 (m, 3H), 1.01-0.95 (m, 6H)

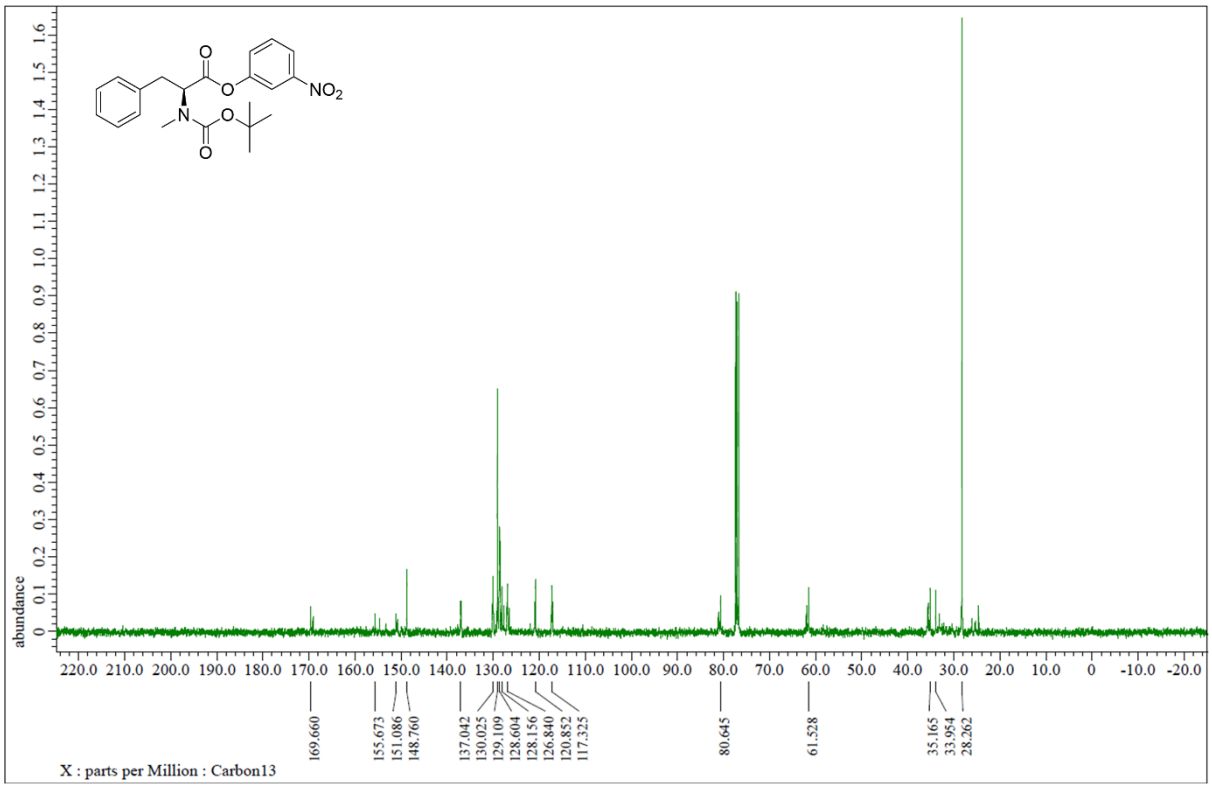


^{13}C NMR (101 MHz, DMSO-d_6) δ 167.4, 149.7, 148.3, 131.1, 128.7, 121.7, 117.2, 58.2, 37.1, 30.9, 24.3, 22.9, 21.4

Supplementary Fig. 39. ^{1}H NMR and ^{13}C NMR spectra of Me-L-Leu-3NP

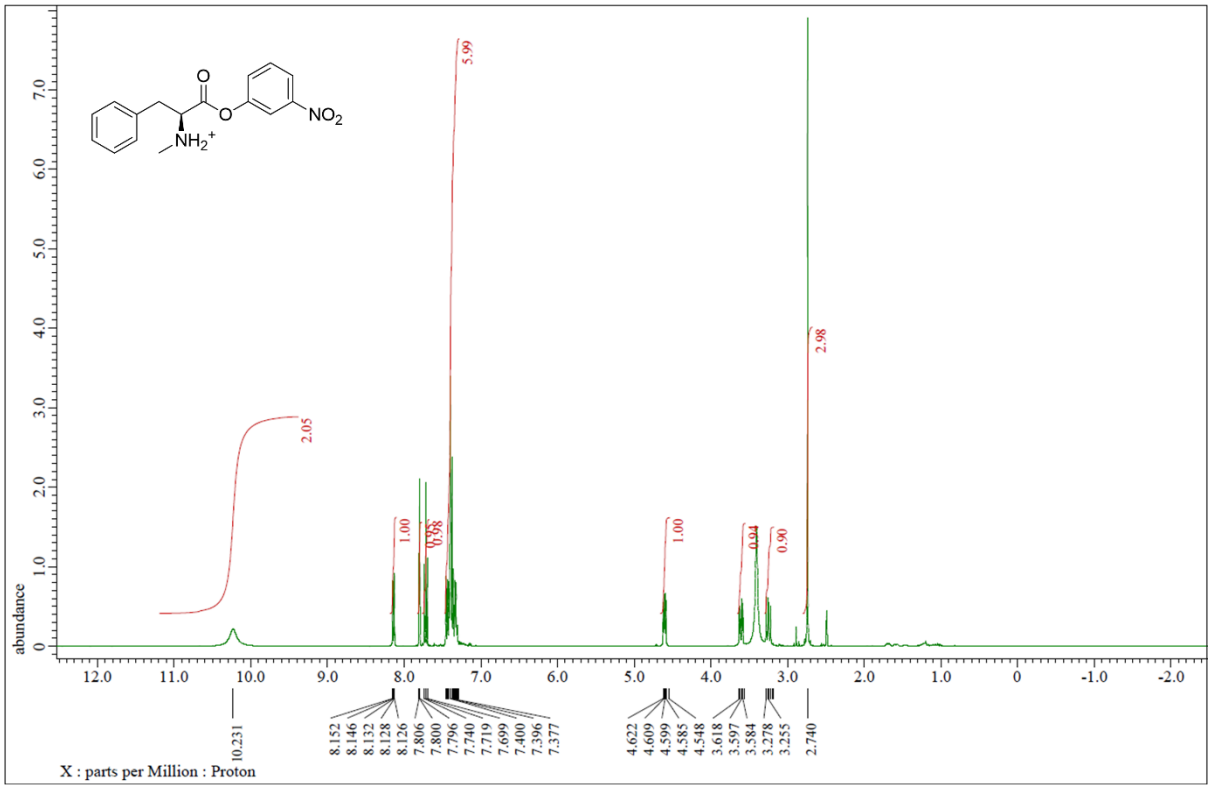


^{1}H NMR (400 MHz, $CDCl_3$) δ 8.12-7.96 (m, 2H), 7.55-7.44 (m, 2H), 7.33-7.26 (m, 5H), 4.78-4.75 (m, 1H), 3.40-3.18 (m, 2H), 2.85-2.77 (m, 3H), 1.45-1.32 (m, 9H)

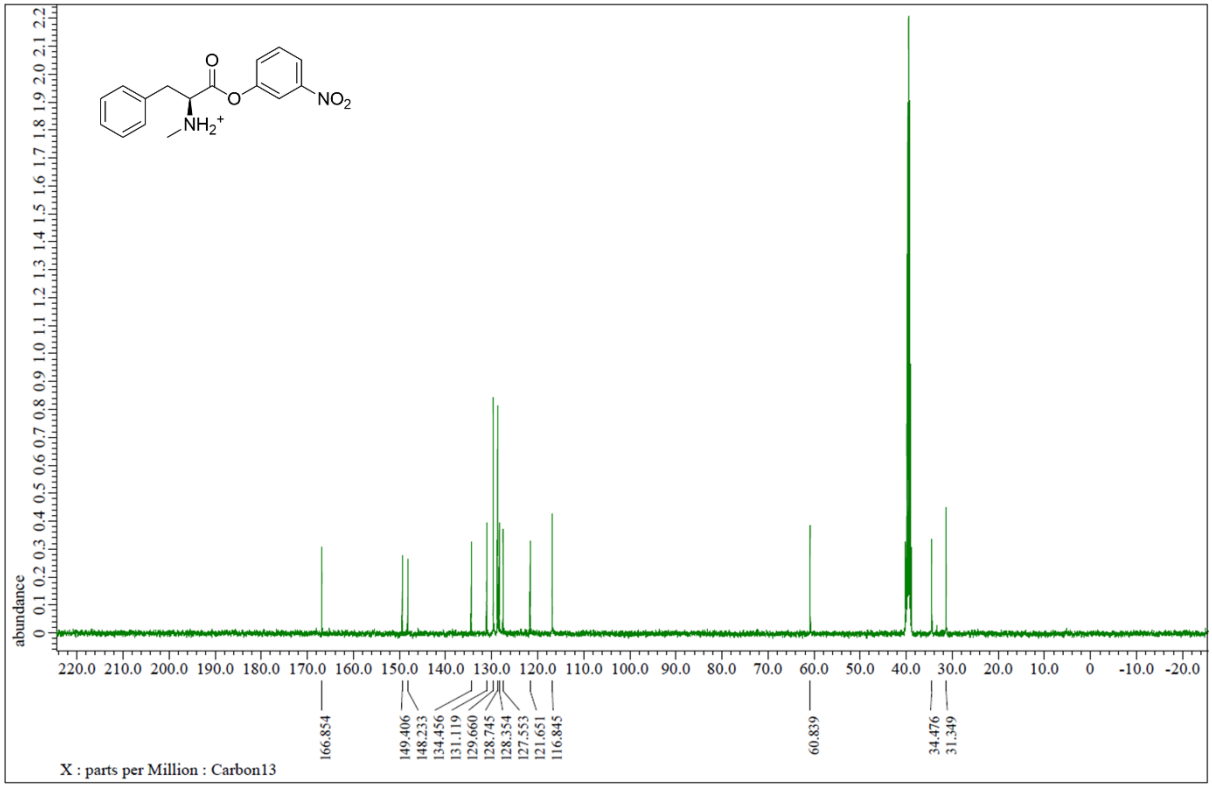


^{13}C NMR (101 MHz, $CDCl_3$) δ 169.7, 155.7, 151.1, 148.8, 137.0, 130.0, 129.1, 128.6, 128.2, 126.8, 120.9, 117.3, 80.6, 61.5, 35.2, 34.0, 28.3

Supplementary Fig. 40. ^{1}H NMR and ^{13}C NMR spectra of Boc-Me-L-Phe-3NP

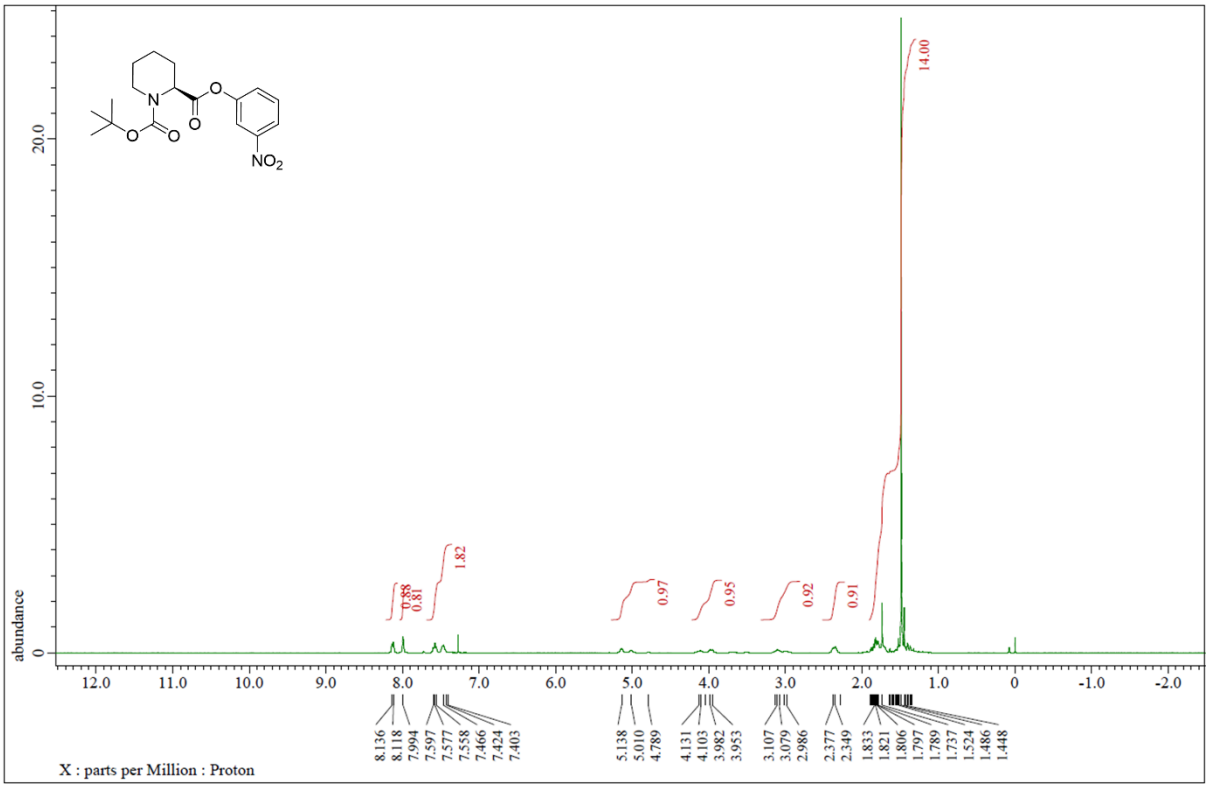


^{1}H NMR (400 MHz, DMSO-d_6) δ 10.23 (s, 2H), 8.15-8.13 (m, 1H), 7.80 (t, J = 2.1 Hz, 1H), 7.72 (t, J = 8.2 Hz, 1H), 7.46-7.29 (m, 6H), 4.62-4.55 (m, 1H), 3.63-3.56 (m, 1H), 3.28-3.19 (m, 1H), 2.74 (s, 3H)

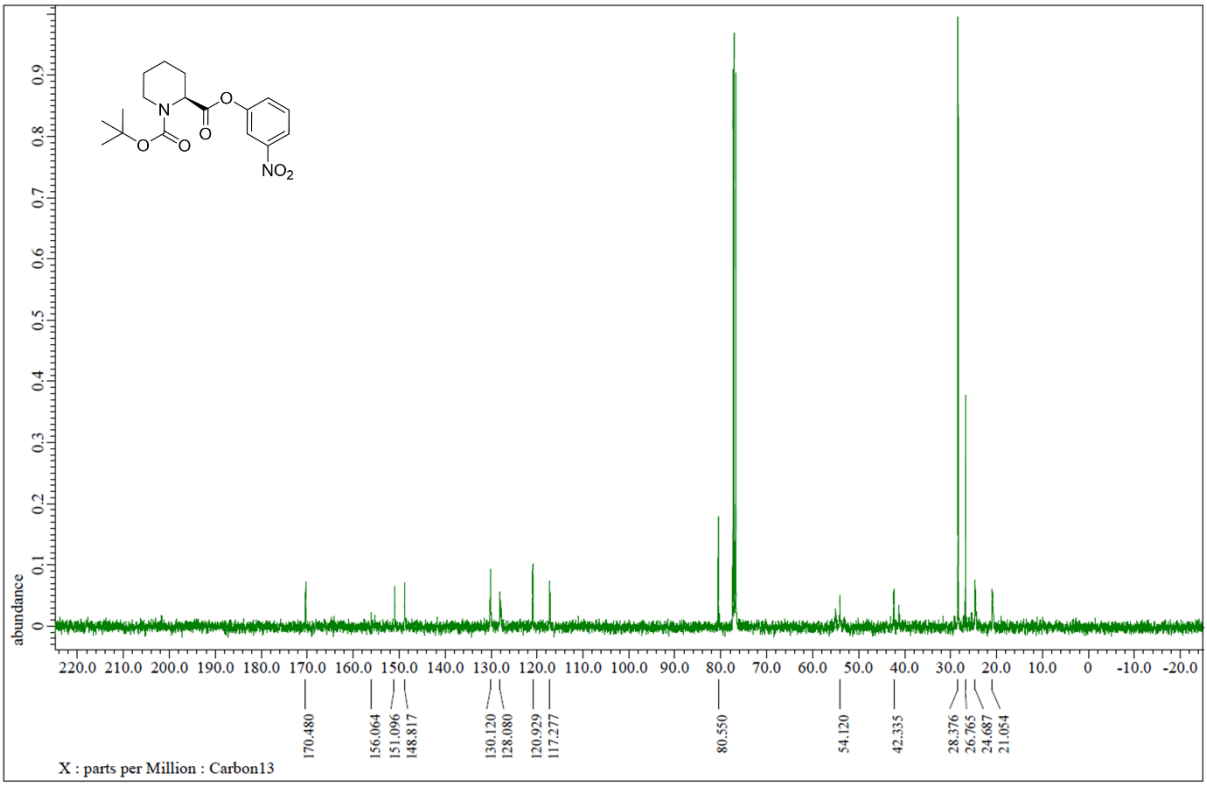


^{13}C NMR (101 MHz, DMSO-d_6) δ 166.9, 149.4, 148.2, 134.5, 131.1, 129.7, 128.7, 128.4, 127.6, 121.7, 116.8, 60.8, 34.5, 31.3

Supplementary Fig. 41. ^{1}H NMR and ^{13}C NMR spectra of Me-L-Phe-3NP

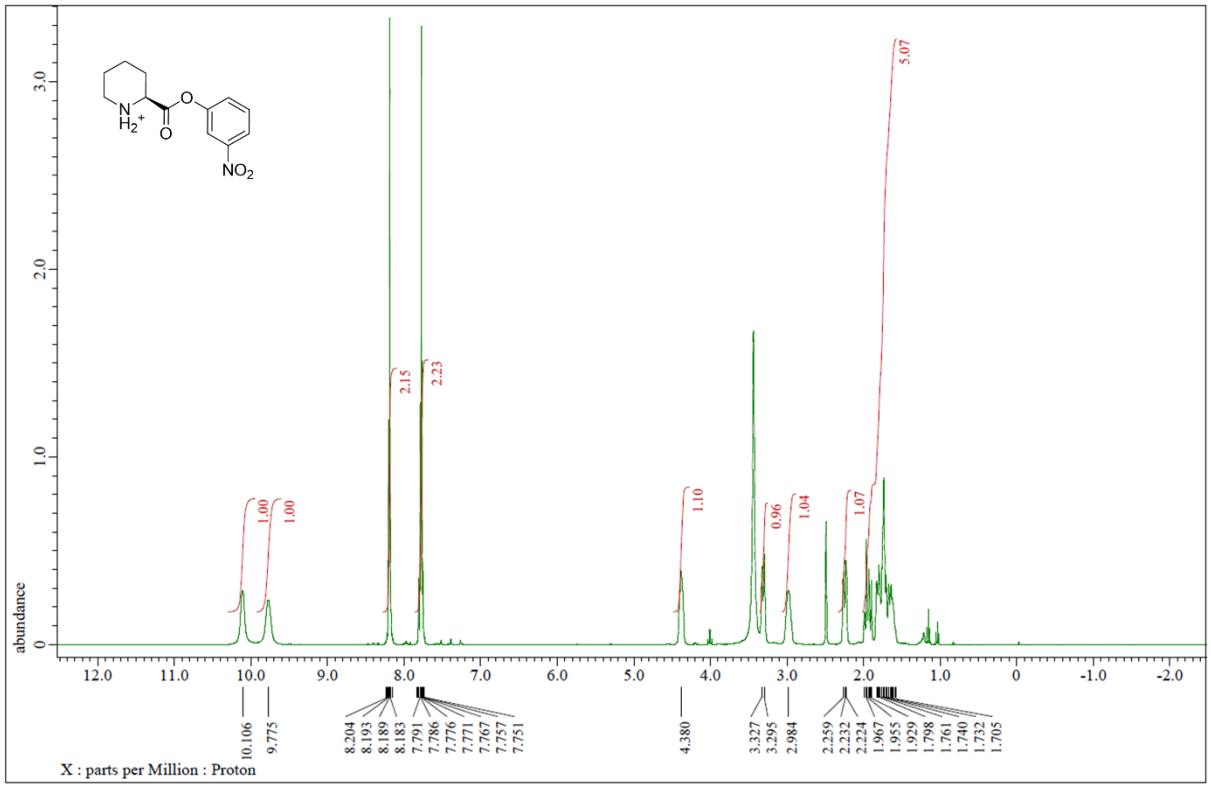


^{1}H NMR (400 MHz, $CDCl_3$) δ 8.13 (d, J = 7.3 Hz, 1H), 7.99 (s, 1H), 7.60-7.40 (m, 2H), 5.14-4.79 (m, 1H), 4.13-3.95 (m, 1H), 3.14-2.99 (m, 1H), 2.38-2.28 (m, 1H), 1.89-1.34 (m, 14H)

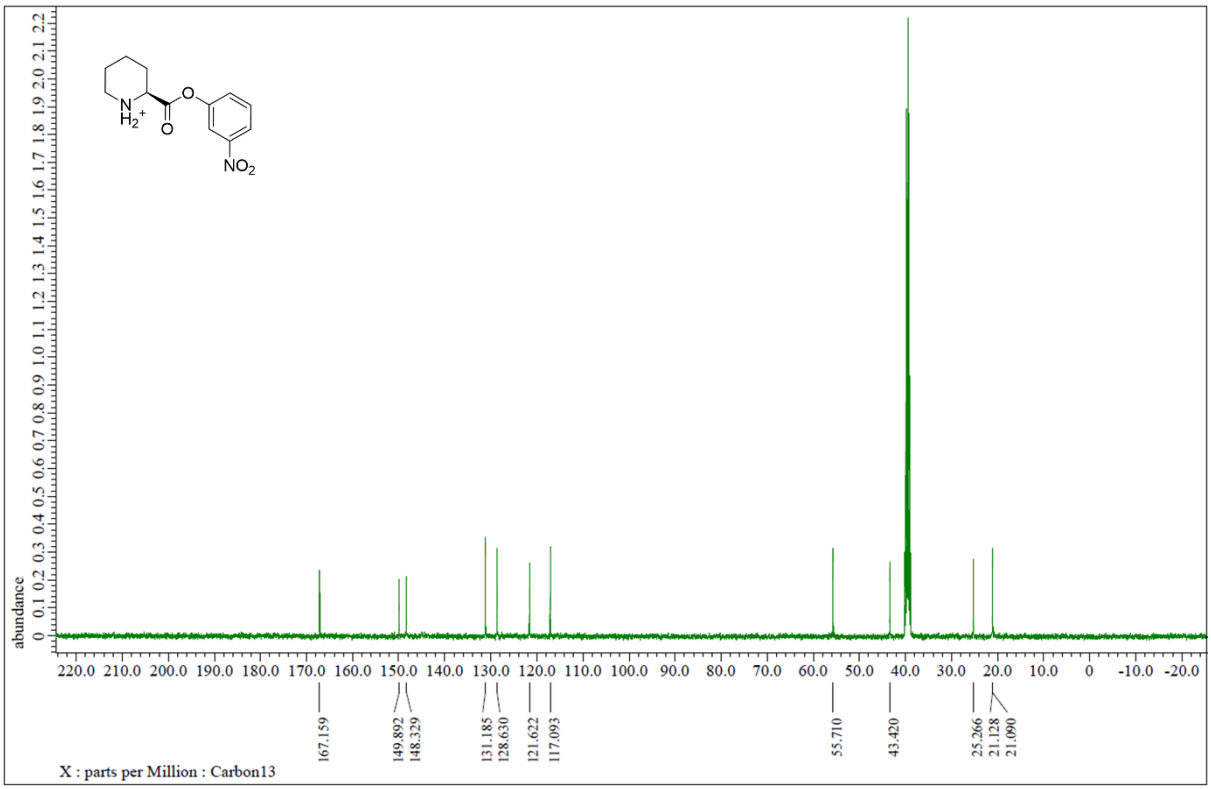


^{13}C NMR (101 MHz, $CDCl_3$) δ 170.5, 156.1, 151.1, 148.8, 130.1, 128.1, 120.9, 117.3, 80.5, 54.1, 42.3, 28.4, 26.8, 24.7, 21.1

Supplementary Fig. 42. ^{1}H NMR and ^{13}C NMR spectra of Boc-Pip-3NP

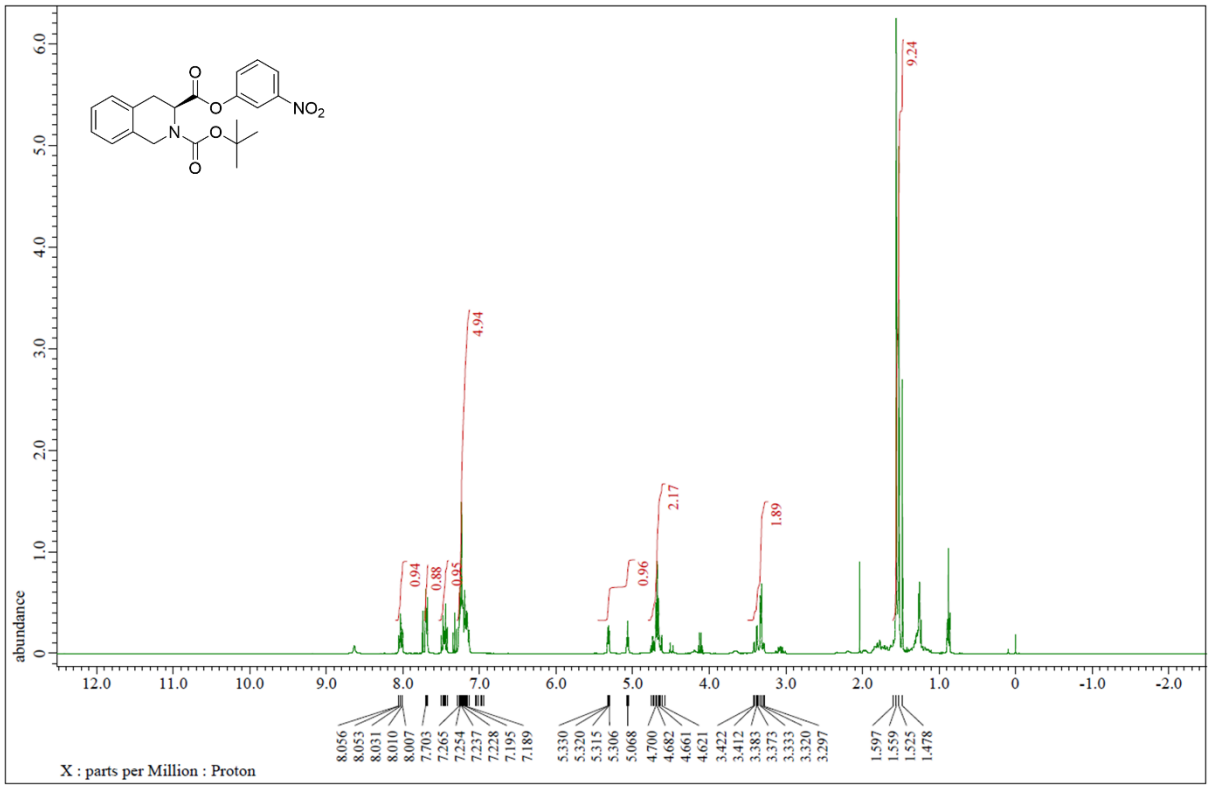


^{1}H NMR (400 MHz, DMSO-d_6) δ 10.11 (s, 1H), 9.78 (s, 1H), 8.24-8.15 (m, 2H), 7.83-7.73 (m, 2H), 4.38 (s, 1H), 3.31 (d, J = 12.8 Hz, 1H), 2.98 (s, 1H), 2.27-2.22 (m, 1H), 1.99-1.57 (m, 5H)

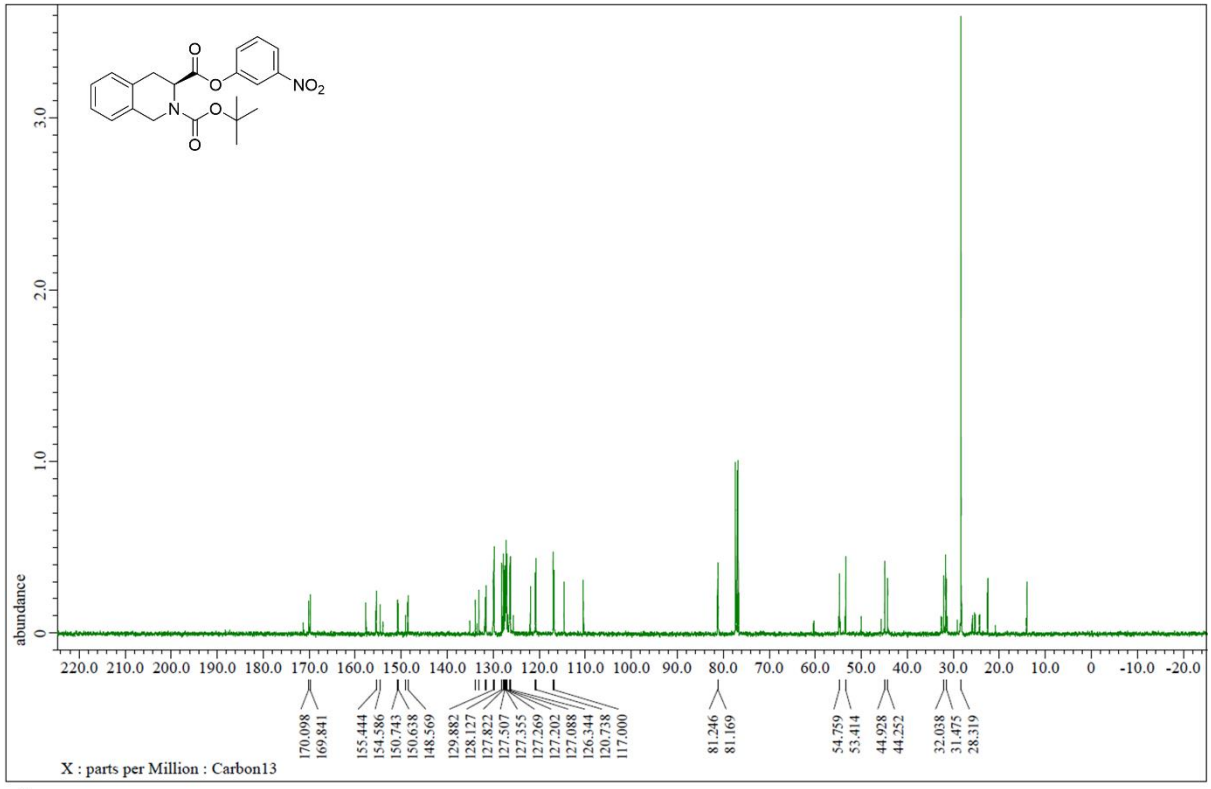


^{13}C NMR (101 MHz, DMSO-d_6) δ 167.2, 149.9, 148.3, 131.2, 128.6, 121.6, 117.1, 55.7, 43.4, 25.3, 21.1, 21.1

Supplementary Fig. 43. ^{1}H NMR and ^{13}C NMR spectra of Pip-3NP

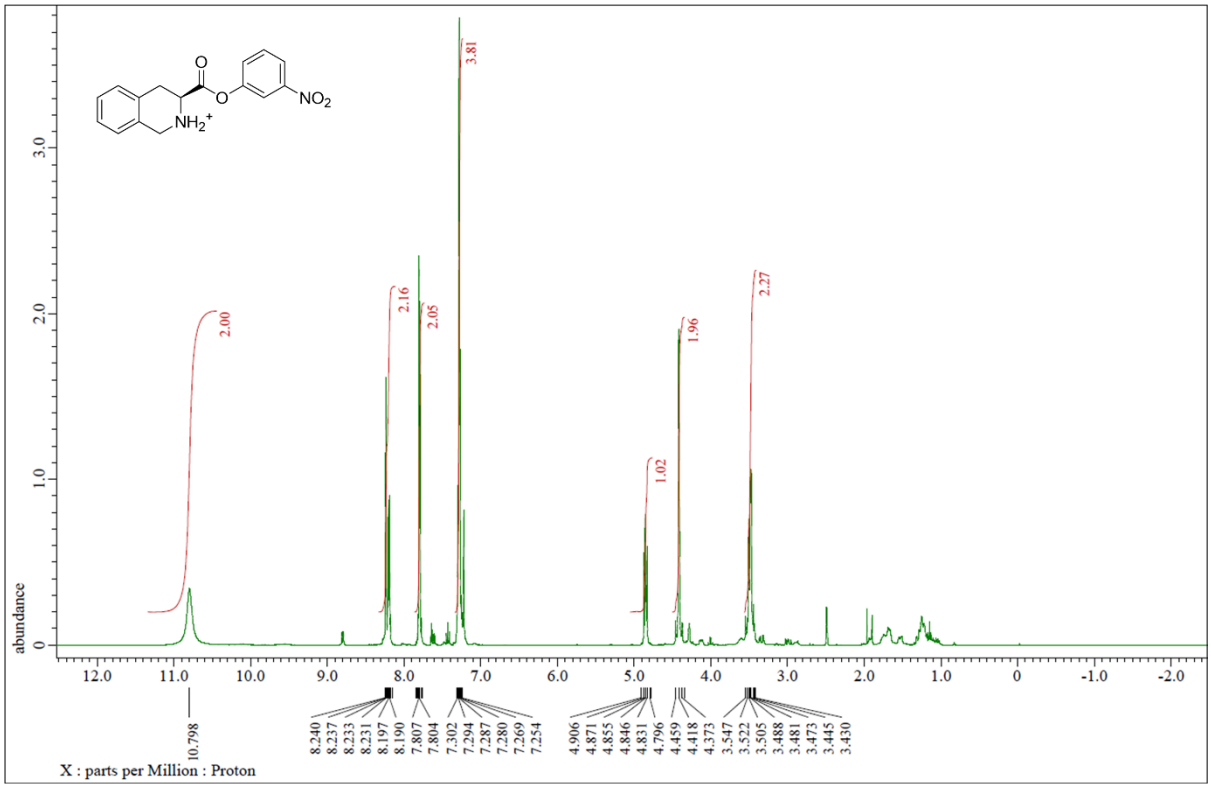


^{1}H NMR (400 MHz, $CDCl_3$) (mixture of rotamers) δ 8.06-8.01 (m, 1H), 7.71-7.68 (m, 1H), 7.50-7.43 (m, 1H), 7.29-7.14 (m, 5H), 5.33-5.06 (m, 1H), 4.76-4.58 (m, 2H), 3.42-3.28 (m, 2H), 1.60-1.48 (m, 9H)

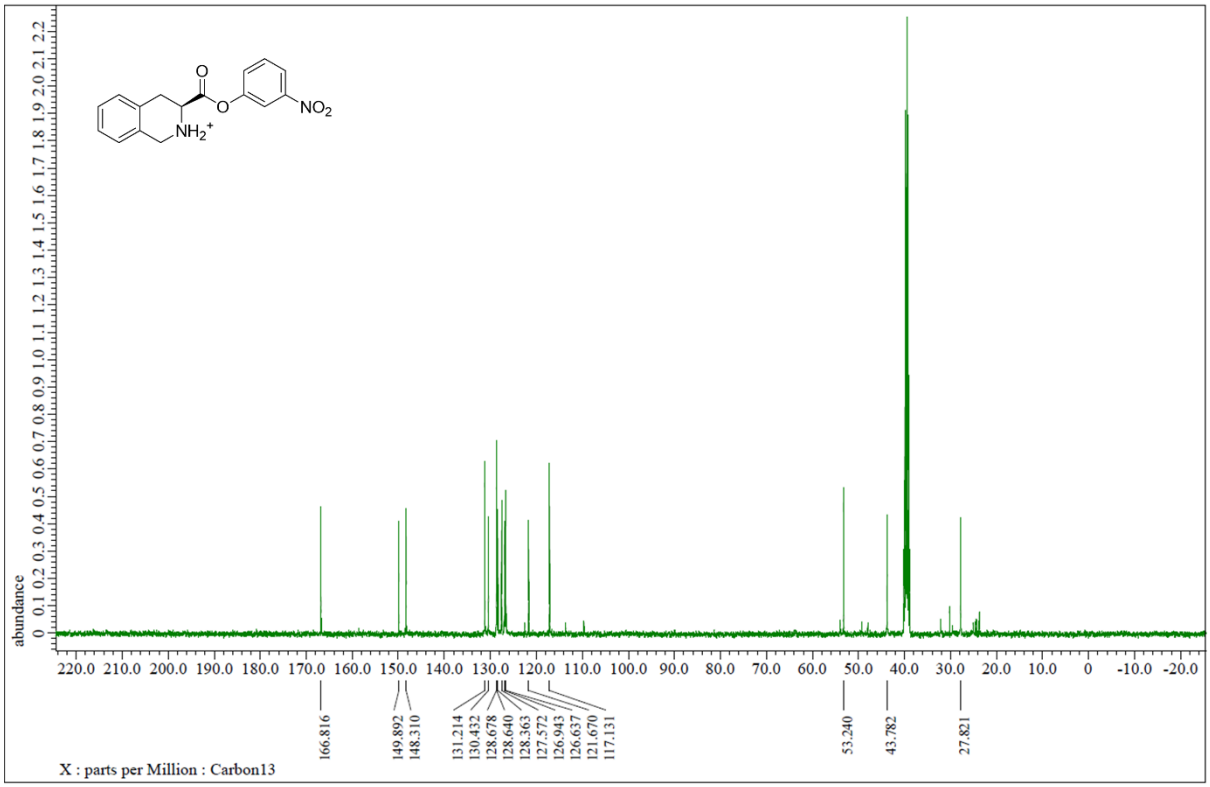


^{13}C NMR (101 MHz, $CDCl_3$) (mixture of rotamers) δ 170.1, 169.8, 155.4, 154.6, 150.7, 150.6, 149.0, 148.6, 133.9, 133.1, 131.8, 131.6, 130.0, 129.9, 128.1, 127.8, 127.6, 127.5, 127.4, 127.3, 127.2, 127.1, 126.5, 126.3, 120.8, 120.7, 117.0, 116.8, 81.2, 81.2, 54.8, 53.4, 44.9, 44.3, 32.0, 31.5, 28.3

Supplementary Fig. 44. ^{1}H NMR and ^{13}C NMR spectra of Boc-Tic-3NP

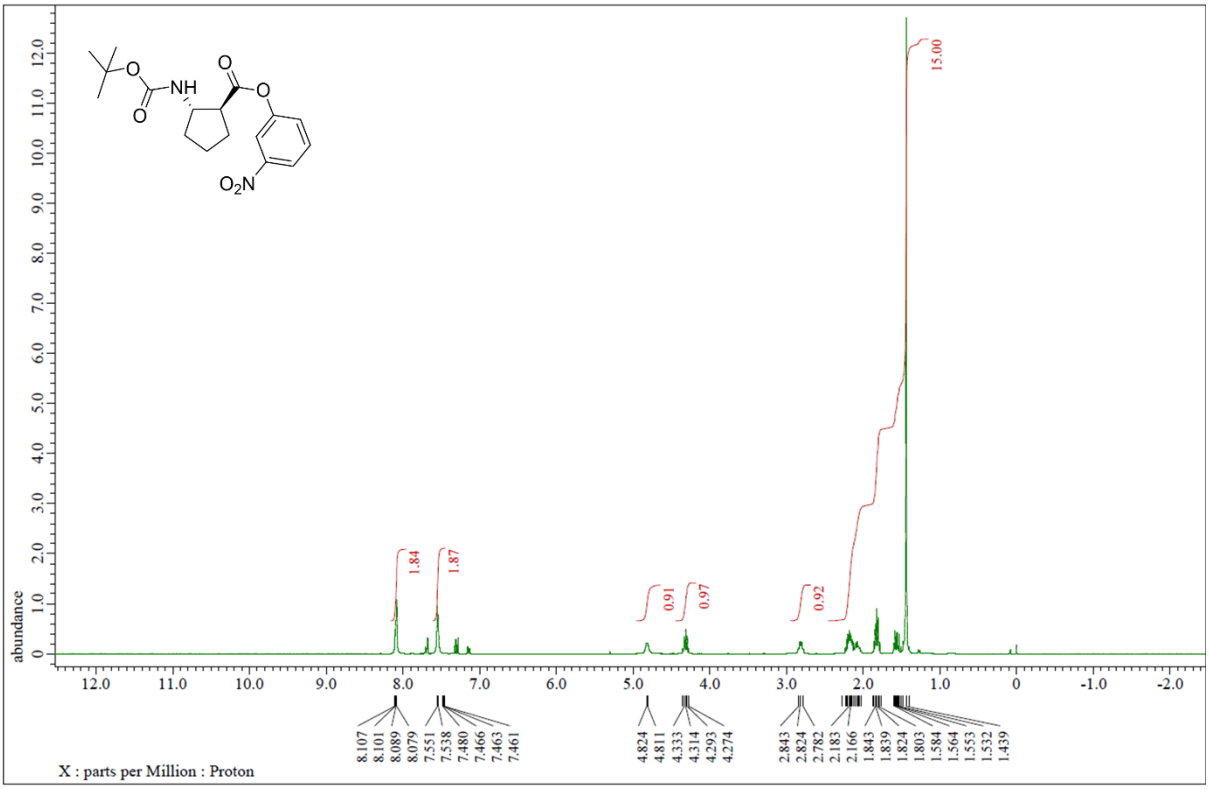


^{1}H NMR (400 MHz, DMSO-d_6) δ 10.80 (s, 2H), 8.24-8.15 (m, 2H), 7.84-7.76 (m, 2H), 7.31-7.24 (m, 4H), 4.91-4.78 (m, 1H), 4.46-4.35 (m, 2H), 3.55-3.42 (m, 2H)

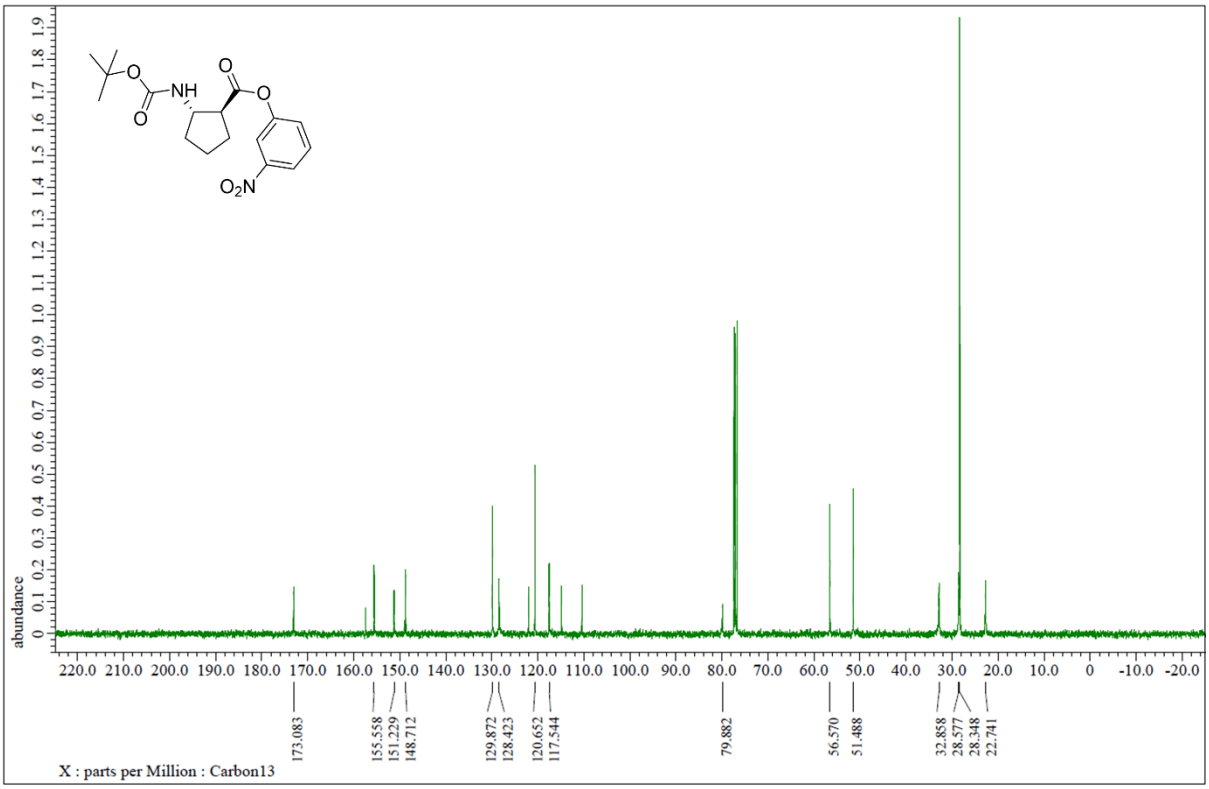


^{13}C NMR (101 MHz, DMSO-d_6) δ 166.8, 149.9, 148.3, 131.2, 130.4, 128.7, 128.6, 128.4, 127.6, 126.9, 126.6, 121.7, 117.1, 53.2, 43.8, 27.8

Supplementary Fig. 45 . ^{1}H NMR and ^{13}C NMR spectra of Tic-3NP

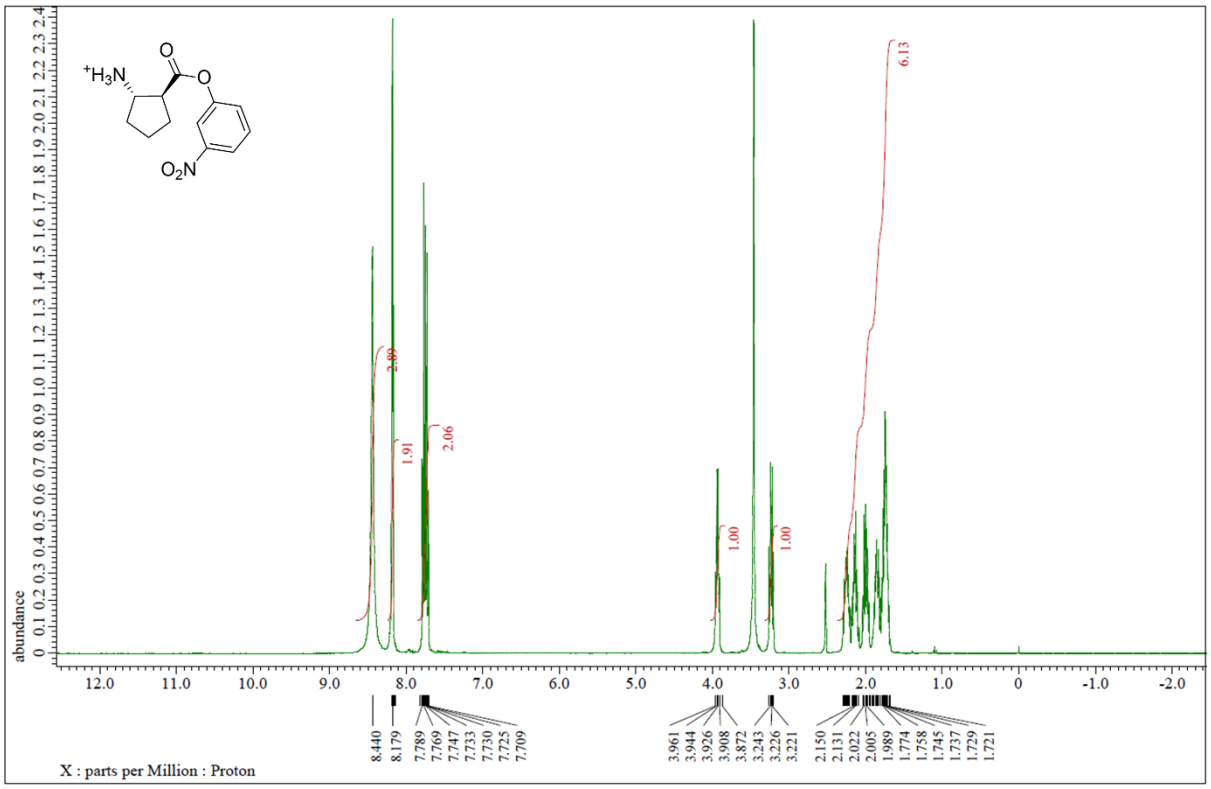


^{1}H NMR (400 MHz, $CDCl_3$) δ 8.11-8.08 (m, 2H), 7.55-7.46 (m, 2H), 4.82 (d, J = 5.0 Hz, 1H), 4.35-4.27 (m, 1H), 2.84-2.78 (m, 1H), 2.27-1.40 (m, 15H)

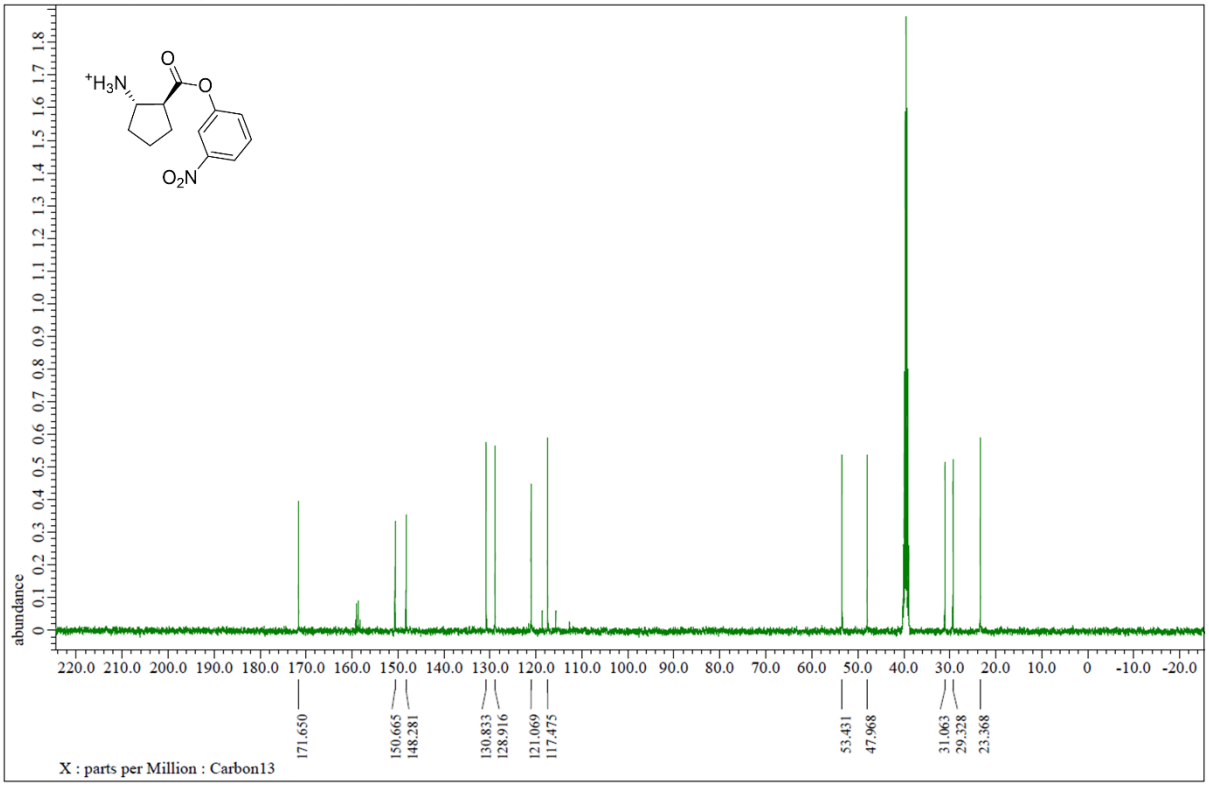


^{13}C NMR (101 MHz, $CDCl_3$) δ 173.1, 155.6, 151.2, 148.7, 129.9, 128.4, 120.7, 117.5, 79.9, 56.6, 51.5, 32.9, 28.6, 28.3, 22.7

Supplementary Fig. 46. ^{1}H NMR and ^{13}C NMR spectra of Boc-(1*S*,2*S*)-2-ACPC-3NP

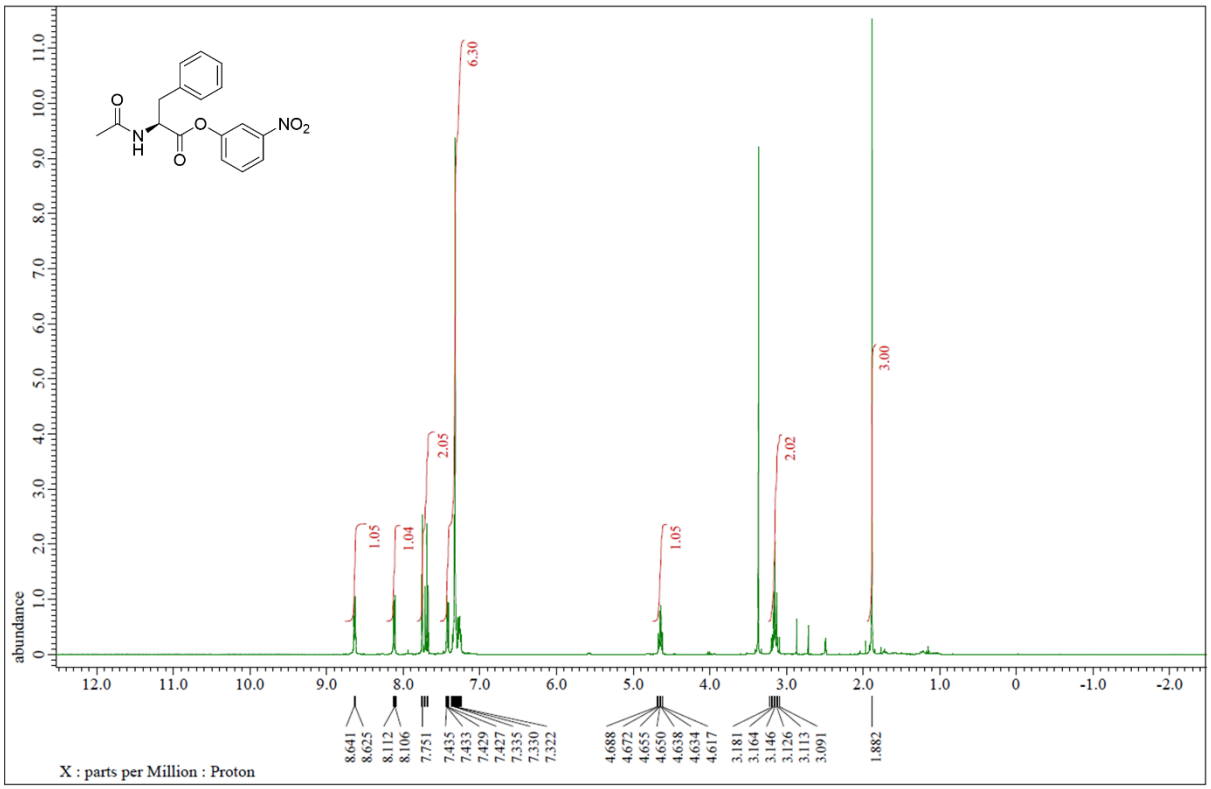


^{1}H NMR (400 MHz, DMSO-d_6) δ 8.44 (s, 3H), 8.19-8.14 (m, 2H), 7.83-7.70 (m, 2H), 3.96-3.87 (m, 1H), 3.26-3.20 (m, 1H), 2.30-1.68 (m, 6H)

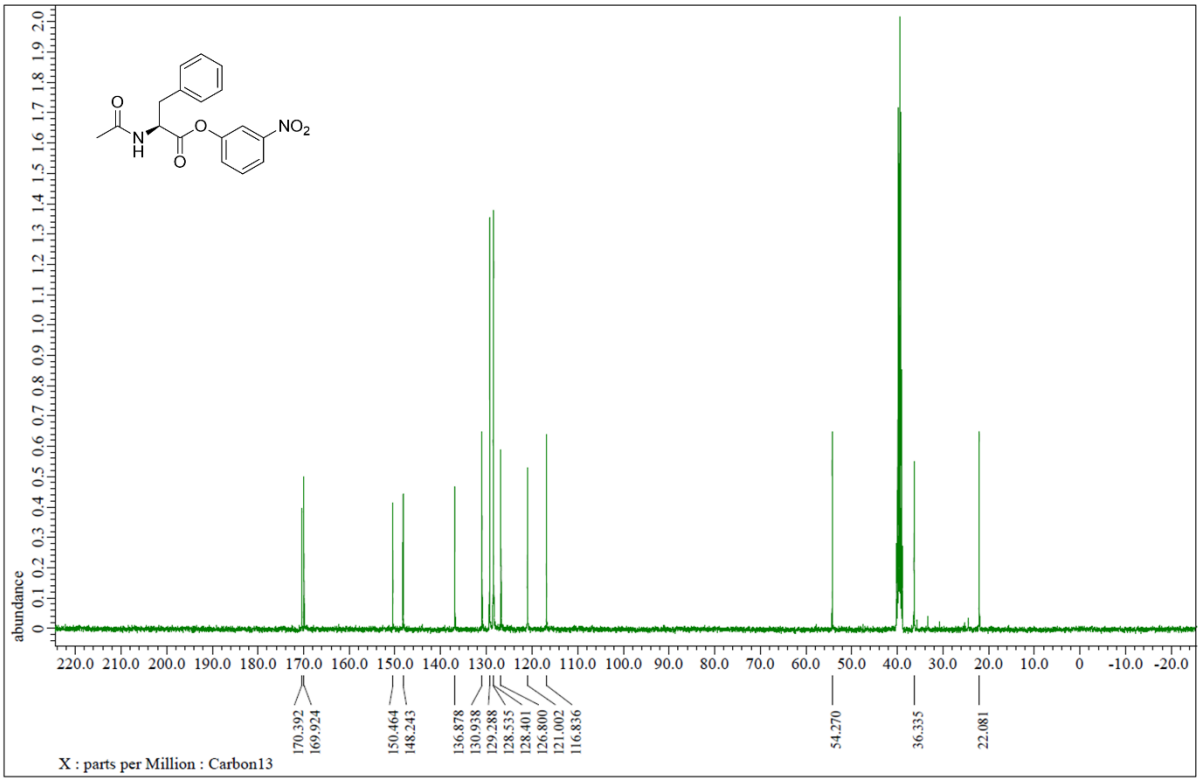


^{13}C NMR (101 MHz, DMSO-d_6) δ 171.7, 150.7, 148.3, 130.8, 128.9, 121.1, 117.5, 53.4, 48.0, 31.1, 29.3, 23.4

Supplementary Fig. 47. ^{1}H NMR and ^{13}C NMR spectra of (1*S*,2*S*)-2-ACPC-3NP

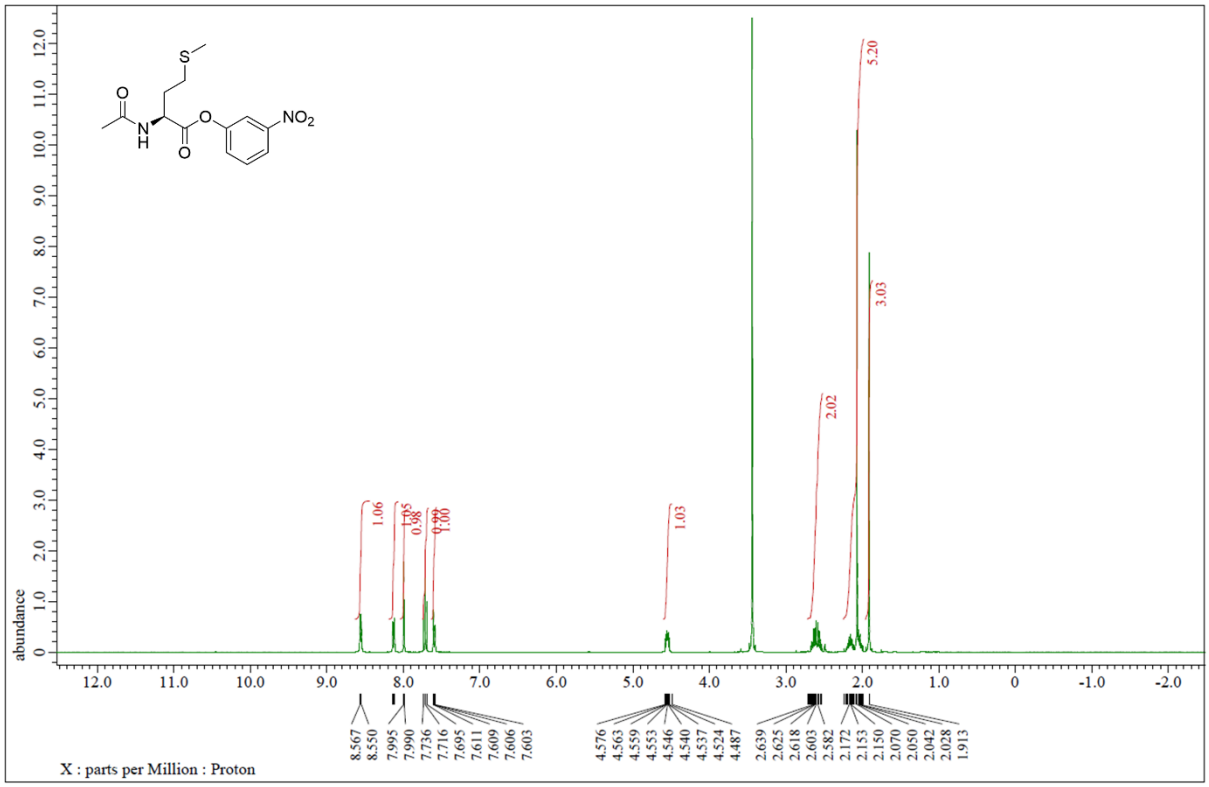


^{1}H NMR (400 MHz, DMSO-d_6) δ 8.63 (d, J = 6.4 Hz, 1H), 8.13-8.09 (m, 1H), 7.76-7.67 (m, 2H), 7.44-7.24 (m, 6H), 4.69-4.62 (m, 1H), 3.22-3.09 (m, 2H), 1.88 (s, 3H)

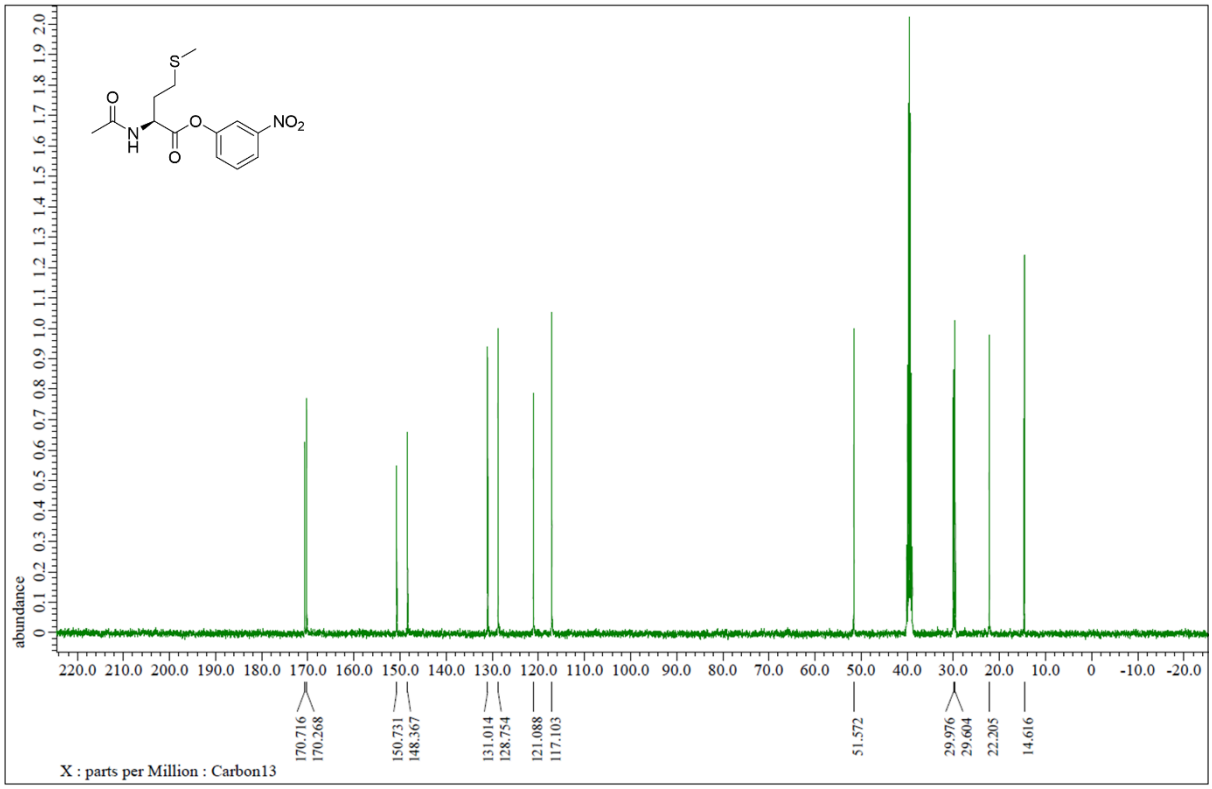


^{13}C NMR (101 MHz, DMSO-d_6) δ 170.4, 169.9, 150.5, 148.2, 136.9, 130.9, 129.3, 128.5, 128.4, 126.8, 121.0, 116.8, 54.3, 36.3, 22.1

Supplementary Fig. 48. ^{1}H NMR and ^{13}C NMR spectra of Ac-L-Phe-3NP

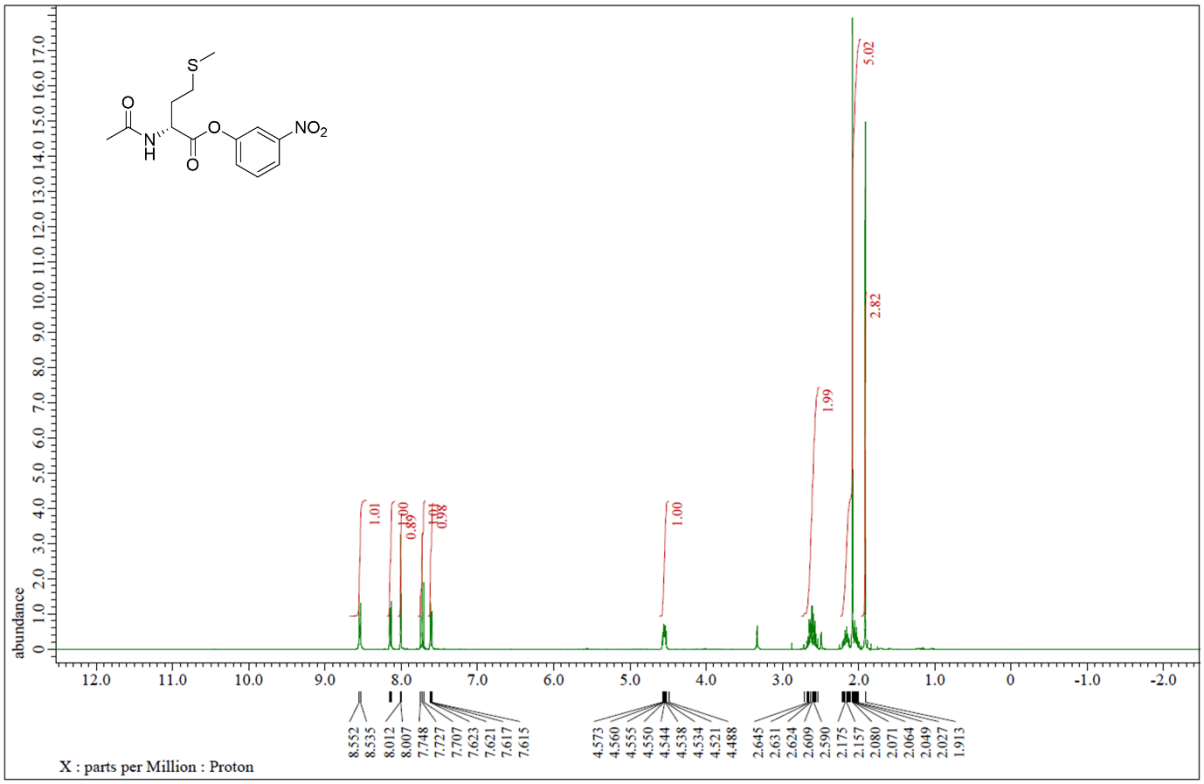


^{1}H NMR (400 MHz, DMSO-d_6) δ 8.56 (d, J = 6.9 Hz, 1H), 8.14-8.11 (m, 1H), 8.00 (t, J = 2.3 Hz, 1H), 7.72 (t, J = 8.2 Hz, 1H), 7.61-7.58 (m, 1H), 4.58-4.52 (m, 1H), 2.71-2.53 (m, 2H), 2.24-1.99 (m, 5H), 1.91 (s, 3H)

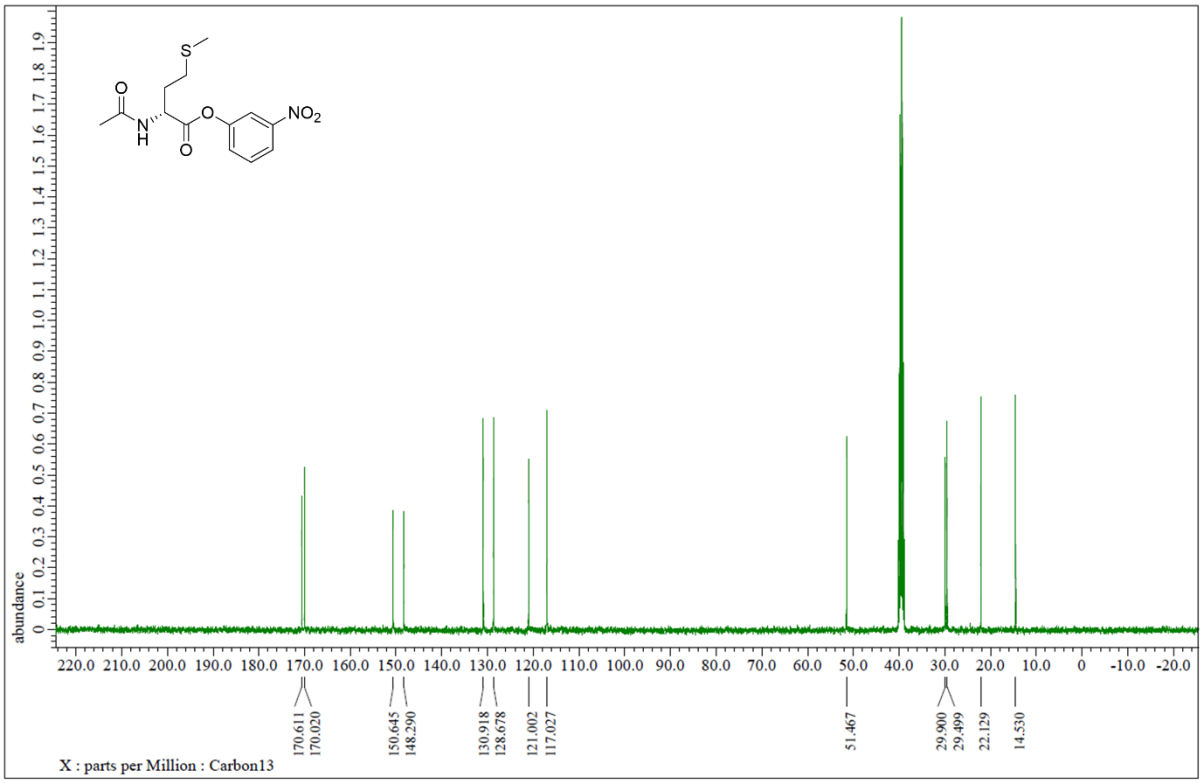


^{13}C NMR (101 MHz, DMSO-d_6) δ 170.7, 170.3, 150.7, 148.4, 131.0, 128.8, 121.1, 117.1, 51.6, 30.0, 29.6, 22.2, 14.6

Supplementary Fig. 49. ^{1}H NMR and ^{13}C NMR spectra of Ac-L-Met-3NP

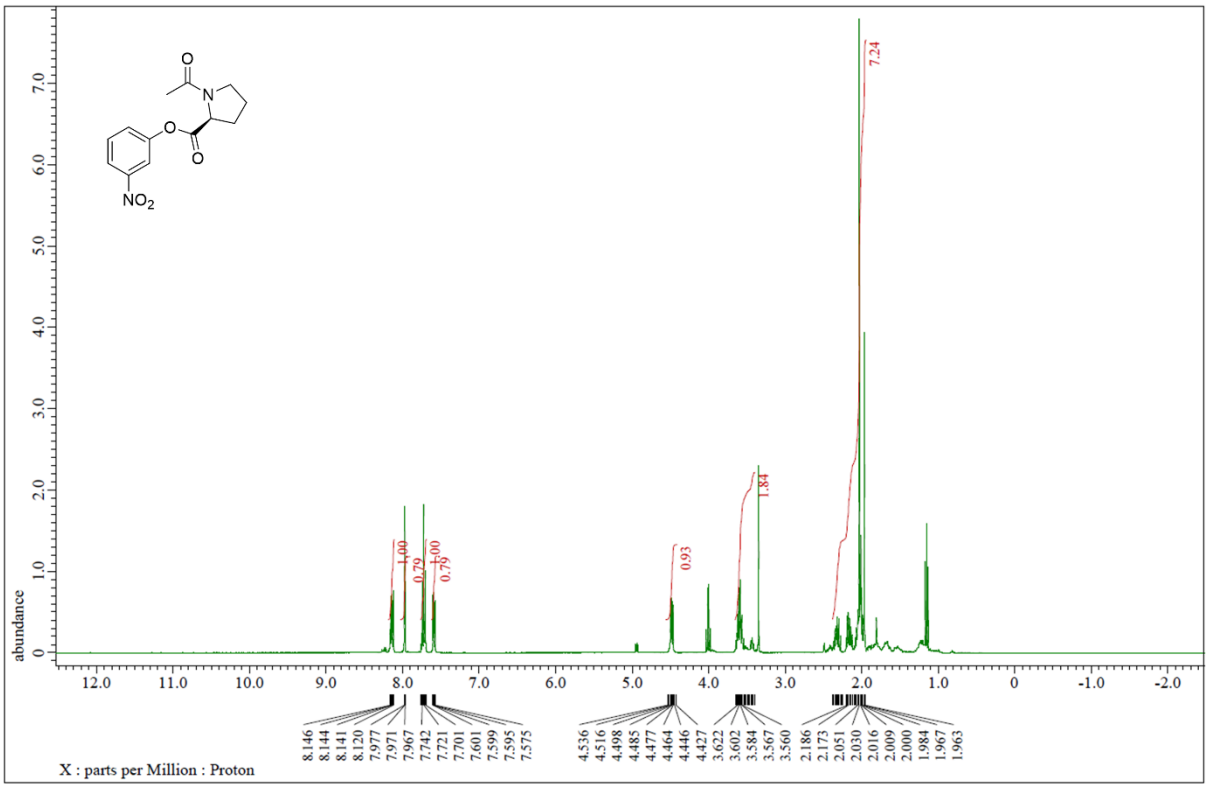


^{1}H NMR (400 MHz, DMSO-d_6) δ 8.54 (d, J = 6.9 Hz, 1H), 8.16-8.13 (m, 1H), 8.01 (t, J = 2.3 Hz, 1H), 7.73 (t, J = 8.2 Hz, 1H), 7.62-7.59 (m, 1H), 4.57-4.52 (m, 1H), 2.72-2.54 (m, 2H), 2.22-2.01 (m, 5H), 1.91 (s, 3H)

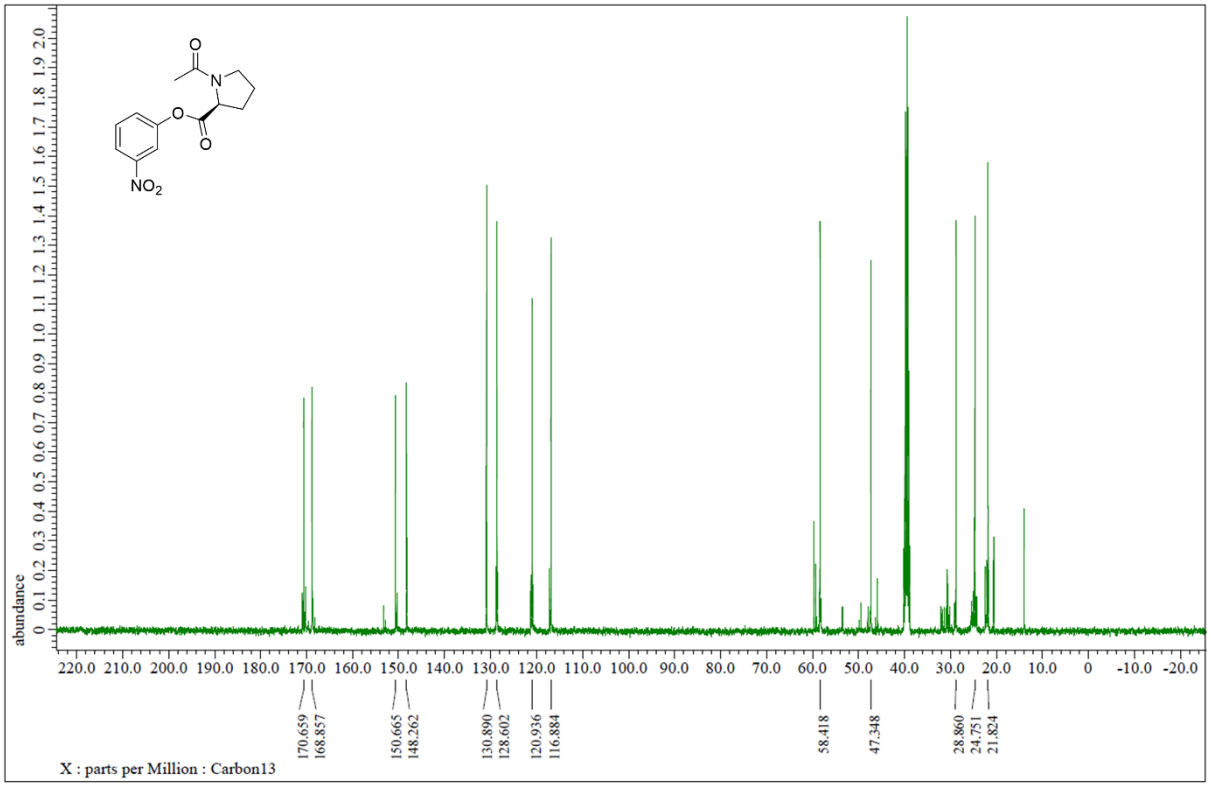


^{13}C NMR (101 MHz, DMSO-d_6) δ 170.6, 170.0, 150.6, 148.3, 130.9, 128.7, 121.0, 117.0, 51.5, 29.9, 29.5, 22.1, 14.5

Supplementary Fig. 50. ^{1}H NMR and ^{13}C NMR spectra of Ac-D-Met-3NP

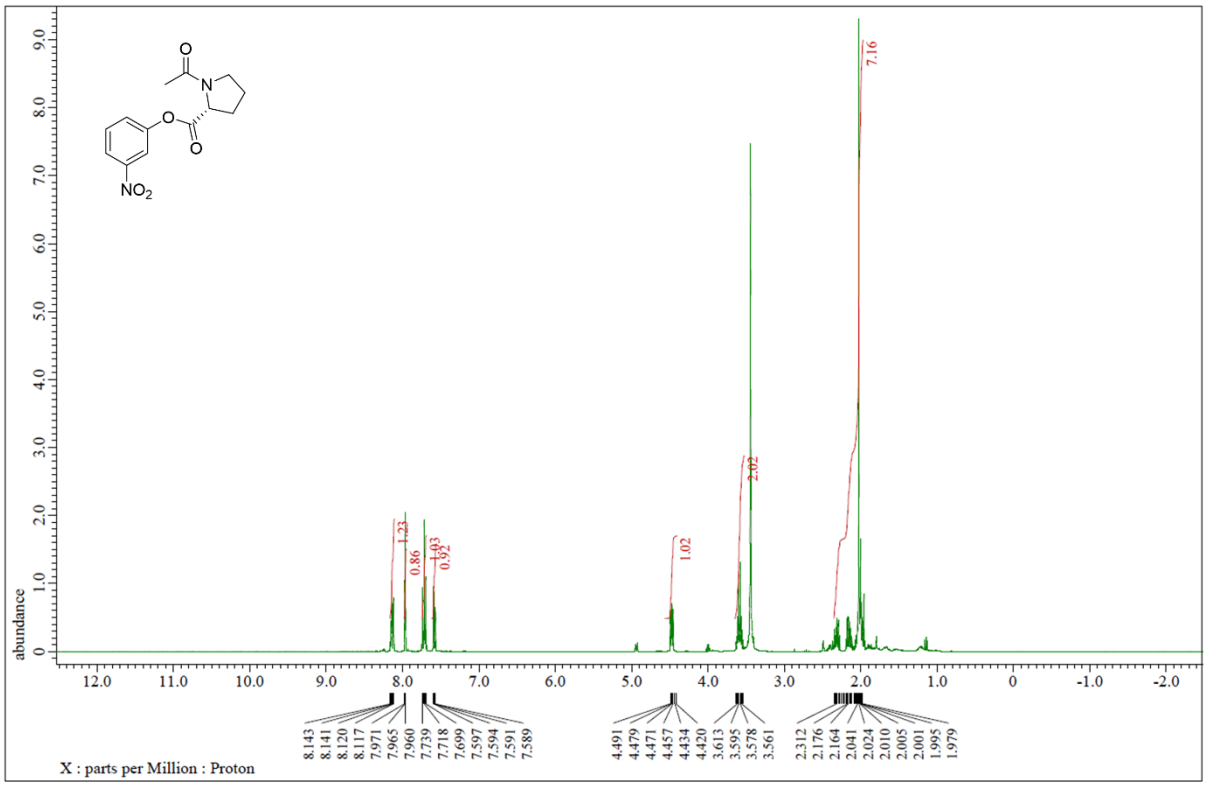


^{1}H NMR (400 MHz, DMSO-d_6) δ 8.16-8.12 (m, 1H), 7.97 (t, J = 2.1 Hz, 1H), 7.76-7.69 (m, 1H), 7.60-7.57 (m, 1H), 4.54-4.43 (m, 1H), 3.65-3.40 (m, 2H), 2.38-1.95 (m, 7H)

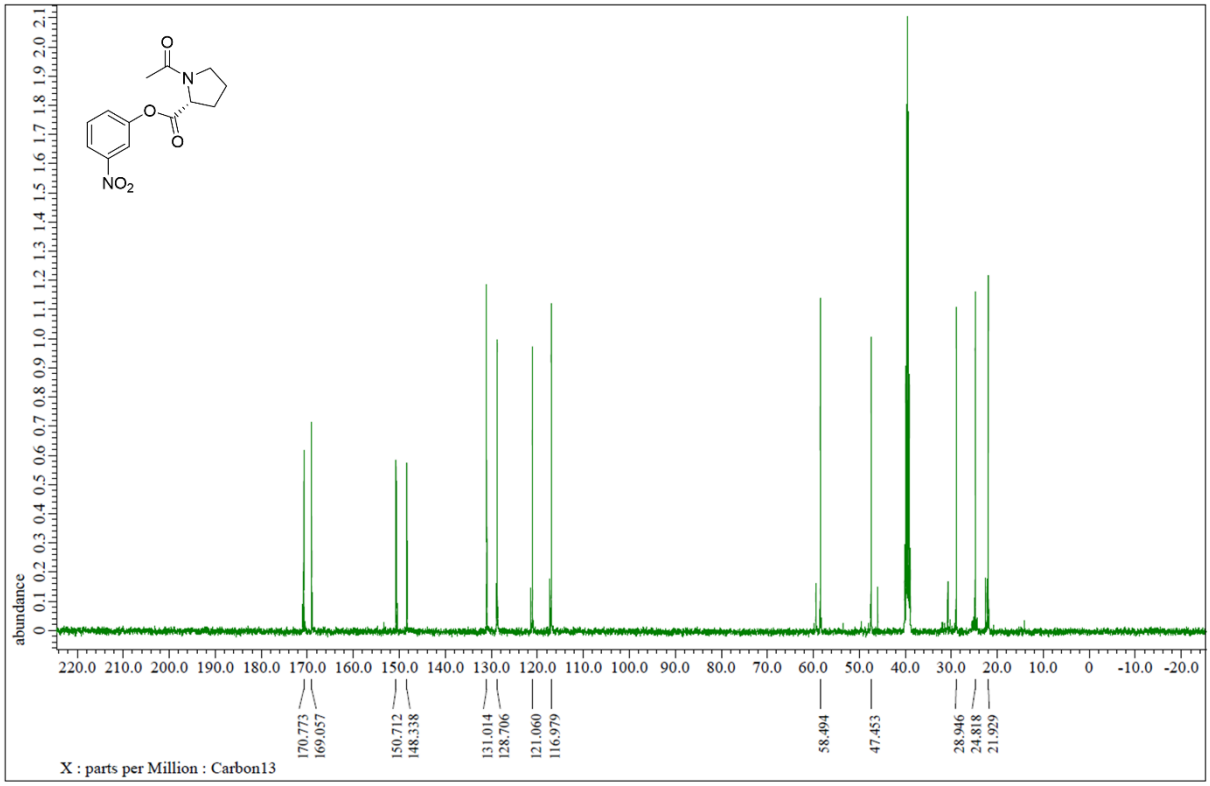


^{13}C NMR (101 MHz, DMSO-d_6) δ 170.7, 168.9, 150.7, 148.3, 130.9, 128.6, 120.9, 116.9, 58.4, 47.3, 28.9, 24.8, 21.8

Supplementary Fig. 51. ^{1}H NMR and ^{13}C NMR spectra of Ac-L-Pro-3NP

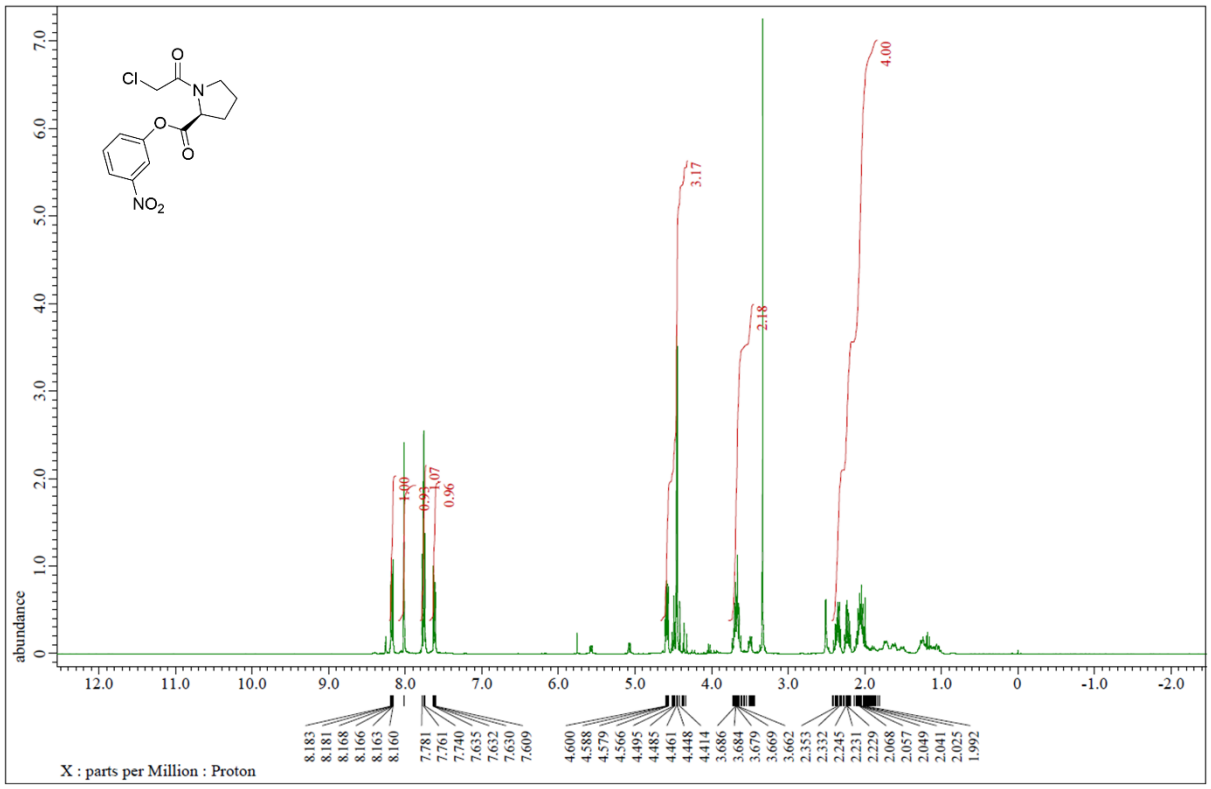


^{1}H NMR (400 MHz, DMSO-d_6) δ 8.16-8.11 (m, 1H), 7.97 (t, J = 2.3 Hz, 1H), 7.74-7.69 (m, 1H), 7.60-7.57 (m, 1H), 4.49-4.42 (m, 1H), 3.64-3.54 (m, 2H), 2.34-1.98 (m, 7H)

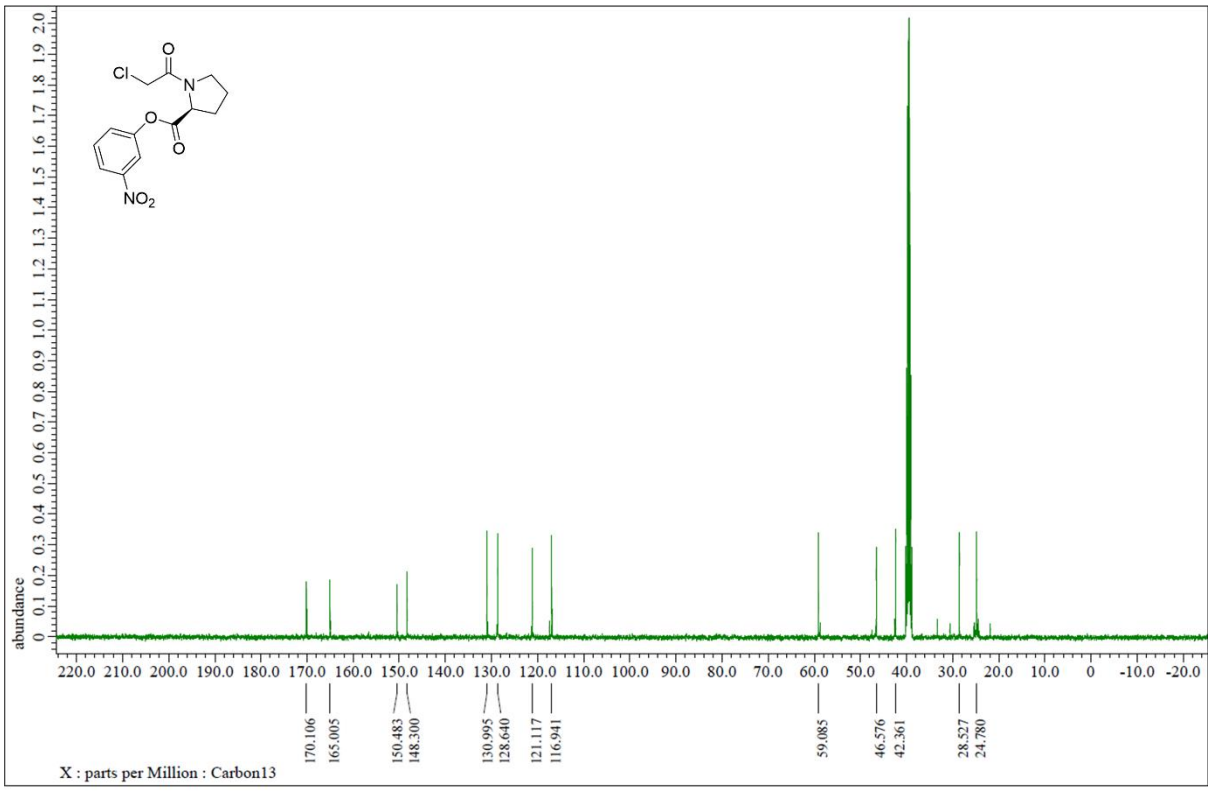


^{13}C NMR (101 MHz, DMSO-d_6) δ 170.8, 169.1, 150.7, 148.3, 131.0, 128.7, 121.1, 117.0, 58.5, 47.5, 28.9, 24.8, 21.9

Supplementary Fig. 52. ^{1}H NMR and ^{13}C NMR spectra of Ac-D-Pro-3NP

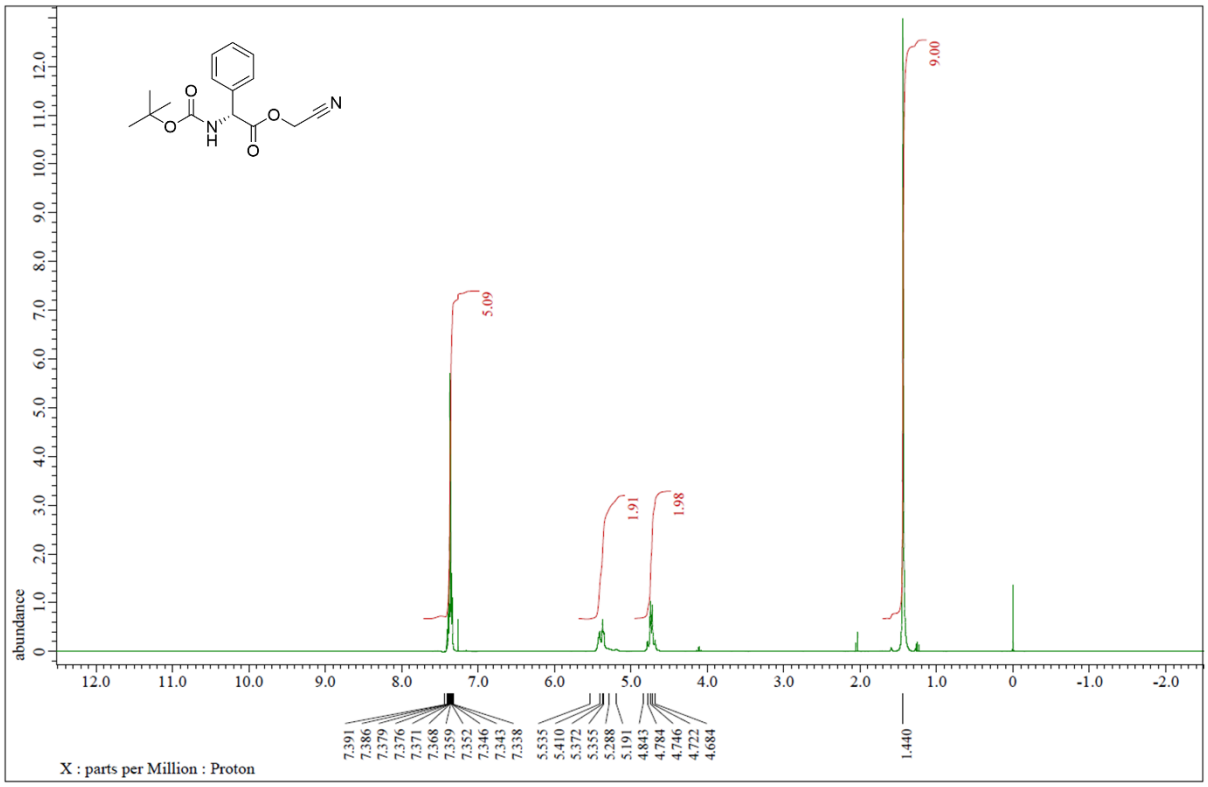


^{1}H NMR (400 MHz, DMSO-d_6) δ 8.19-8.16 (m, 1H), 8.02 (t, J = 2.3 Hz, 1H), 7.76 (t, J = 8.2 Hz, 1H), 7.63-7.61 (m, 1H), 4.60-4.34 (m, 3H), 3.73-3.45 (m, 2H), 2.42-1.85 (m, 4H)

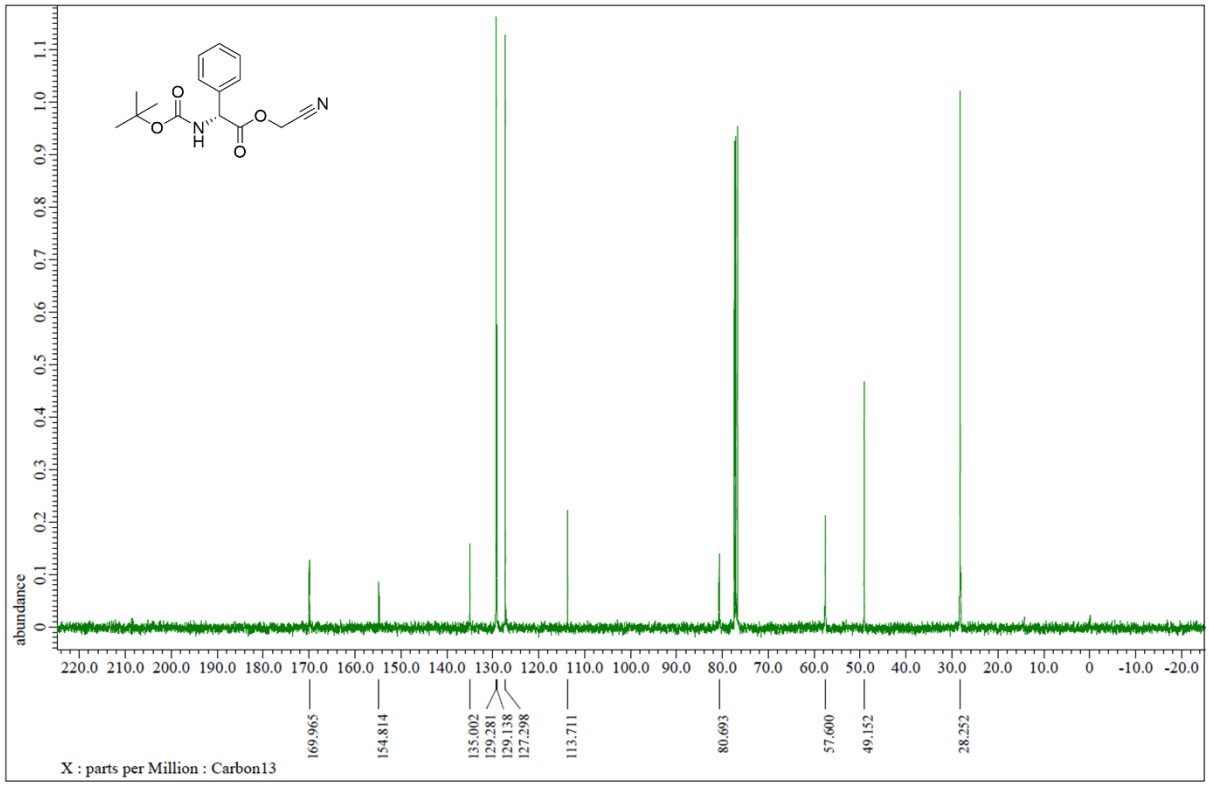


^{13}C NMR (101 MHz, DMSO-d_6) δ 170.1, 165.0, 150.5, 148.3, 131.0, 128.6, 121.1, 116.9, 59.1, 46.6, 42.4, 28.5, 24.8

Supplementary Fig. 53. ^{1}H NMR and ^{13}C NMR spectra of ClAc-L-Pro-3NP

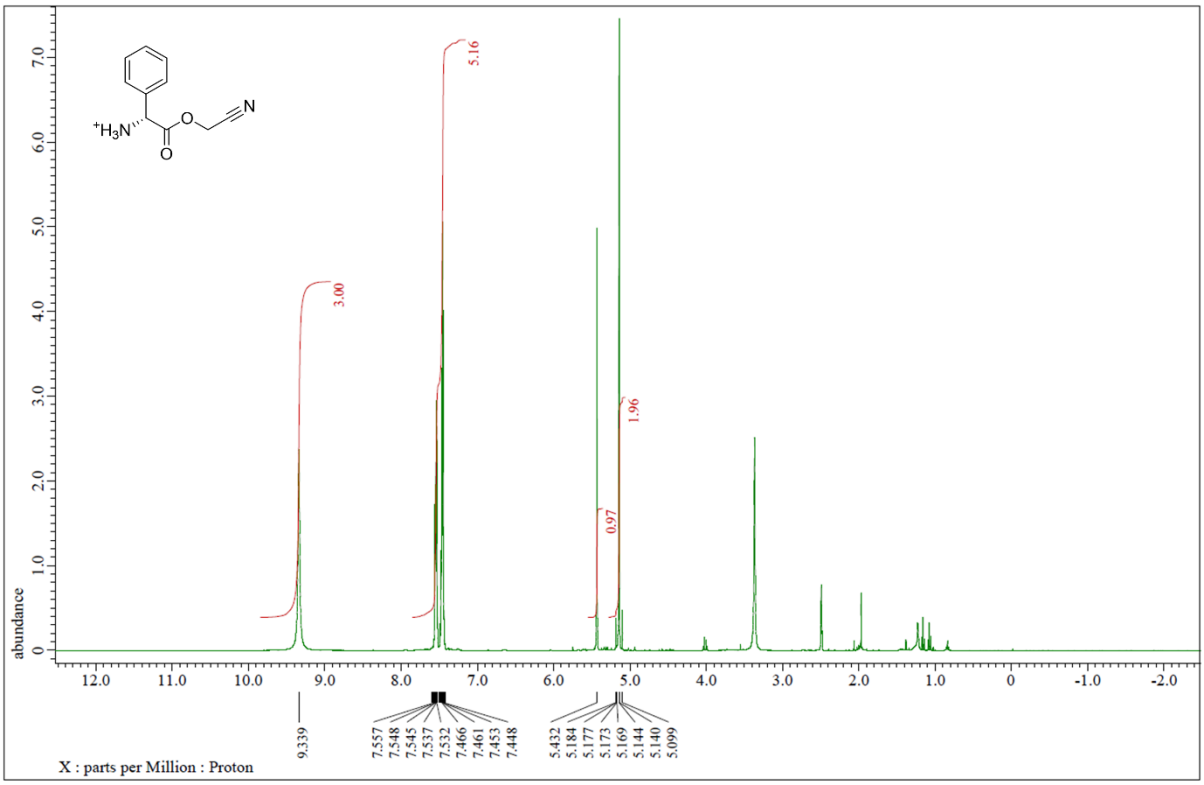


^{1}H NMR (400 MHz, $CDCl_3$) δ 7.44-7.33 (m, 5H), 5.54-5.19 (m, 2H), 4.84-4.68 (m, 2H), 1.44 (s, 9H)

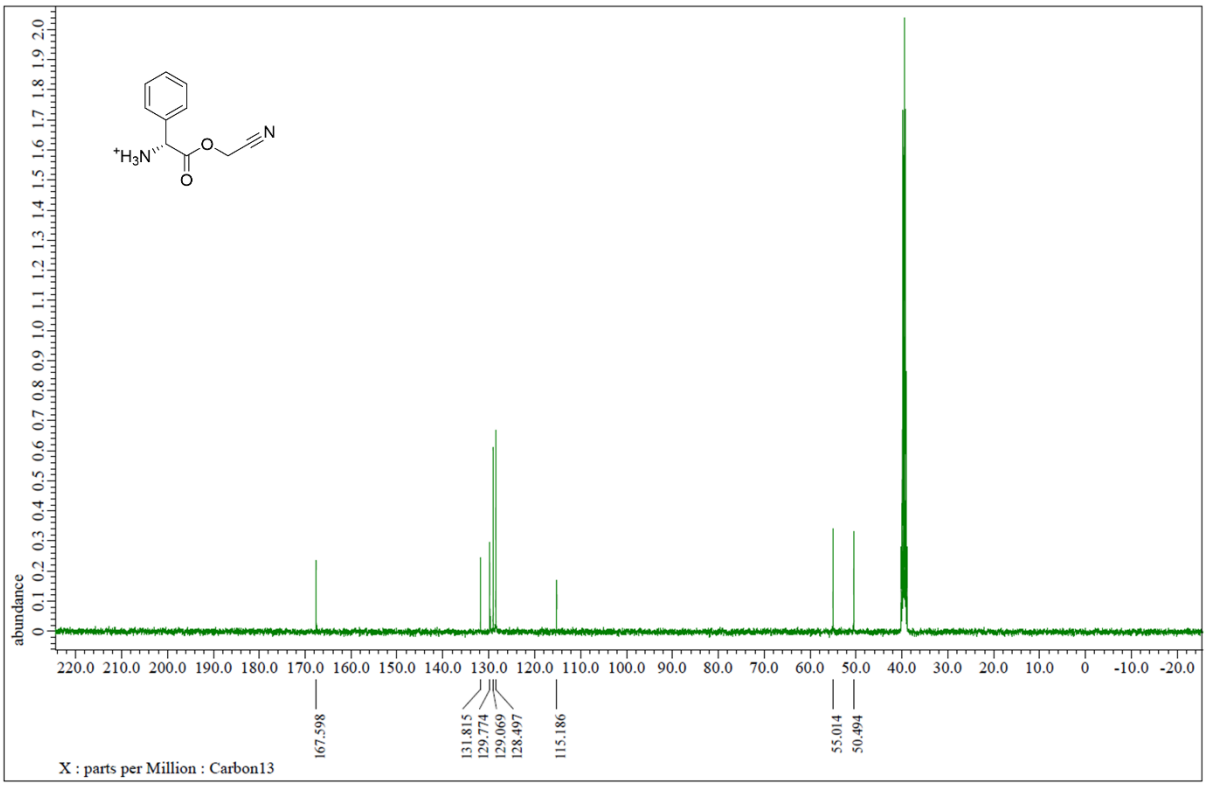


^{13}C NMR (101 MHz, $CDCl_3$) δ 170.0, 154.8, 135.0, 129.3, 129.1, 127.3, 113.7, 80.7, 57.6, 49.2, 28.3

Supplementary Fig. 54. ^{1}H NMR and ^{13}C NMR spectra of Boc-D-Phg-CME

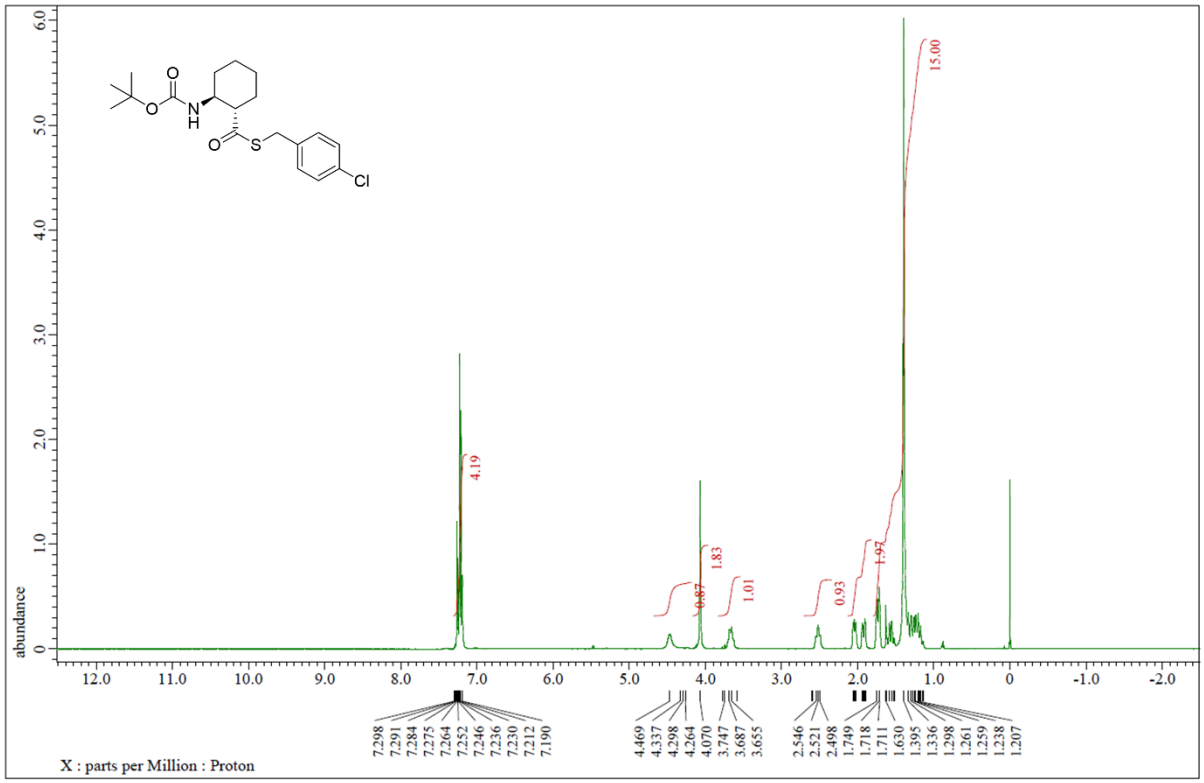


^{1}H NMR (400 MHz, DMSO-d_6) δ 9.34 (s, 3H), 7.60-7.42 (m, 5H), 5.43 (s, 1H), 5.18-5.10 (m, 2H)

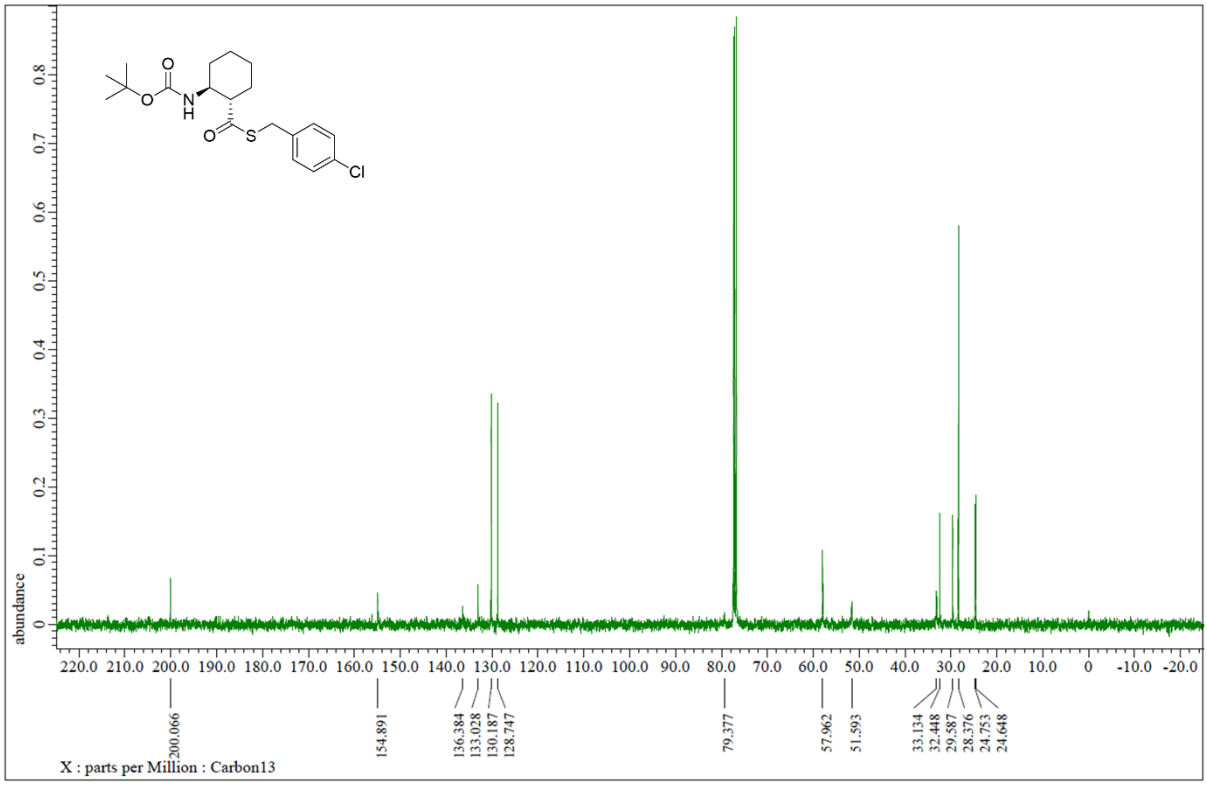


^{13}C NMR (101 MHz, DMSO-d_6) δ 167.6, 131.8, 129.8, 129.1, 128.5, 115.2, 55.0, 50.5

Supplementary Fig. 55. ^{1}H NMR and ^{13}C NMR spectra of D-Phg-CME

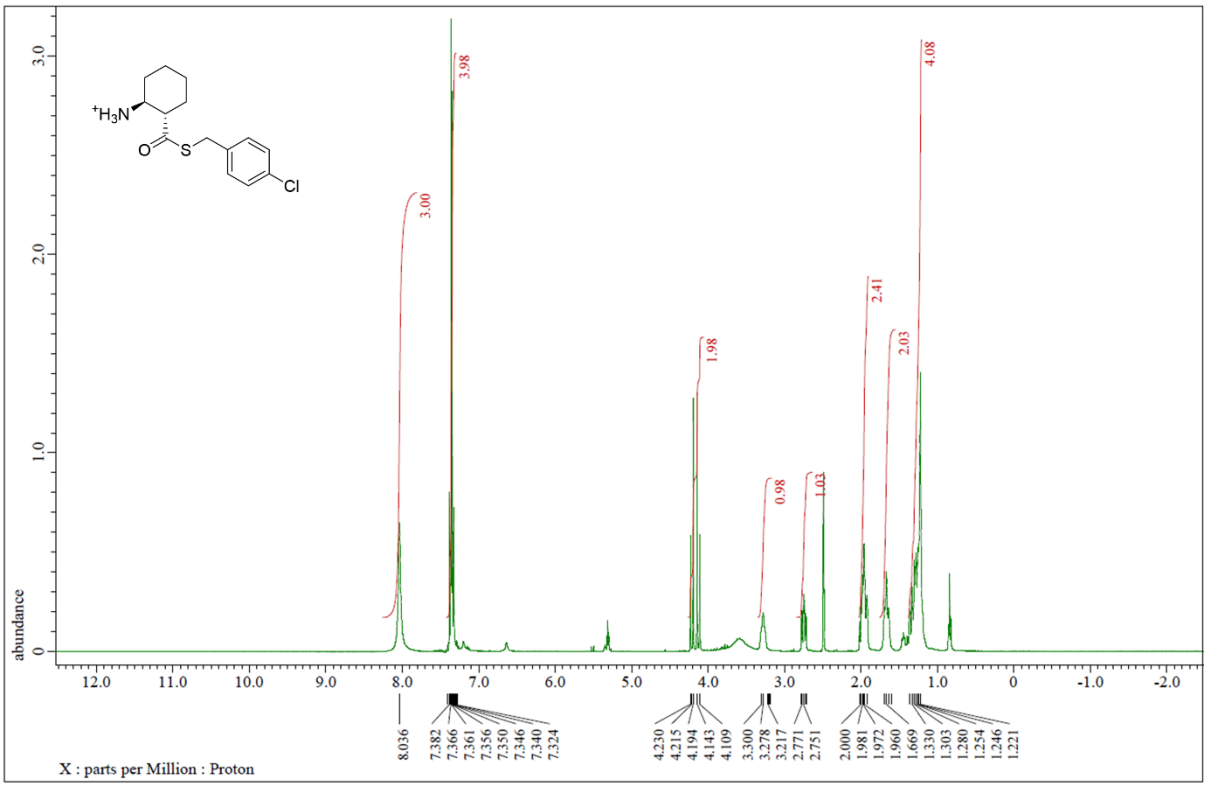


^{1}H NMR (400 MHz, $CDCl_3$) δ 7.30-7.19 (m, 4H), 4.47-4.26 (m, 1H), 4.07 (s, 2H), 3.78-3.58 (m, 1H), 2.60-2.50 (m, 1H), 2.06-1.90 (m, 2H), 1.75-1.14 (m, 15H)

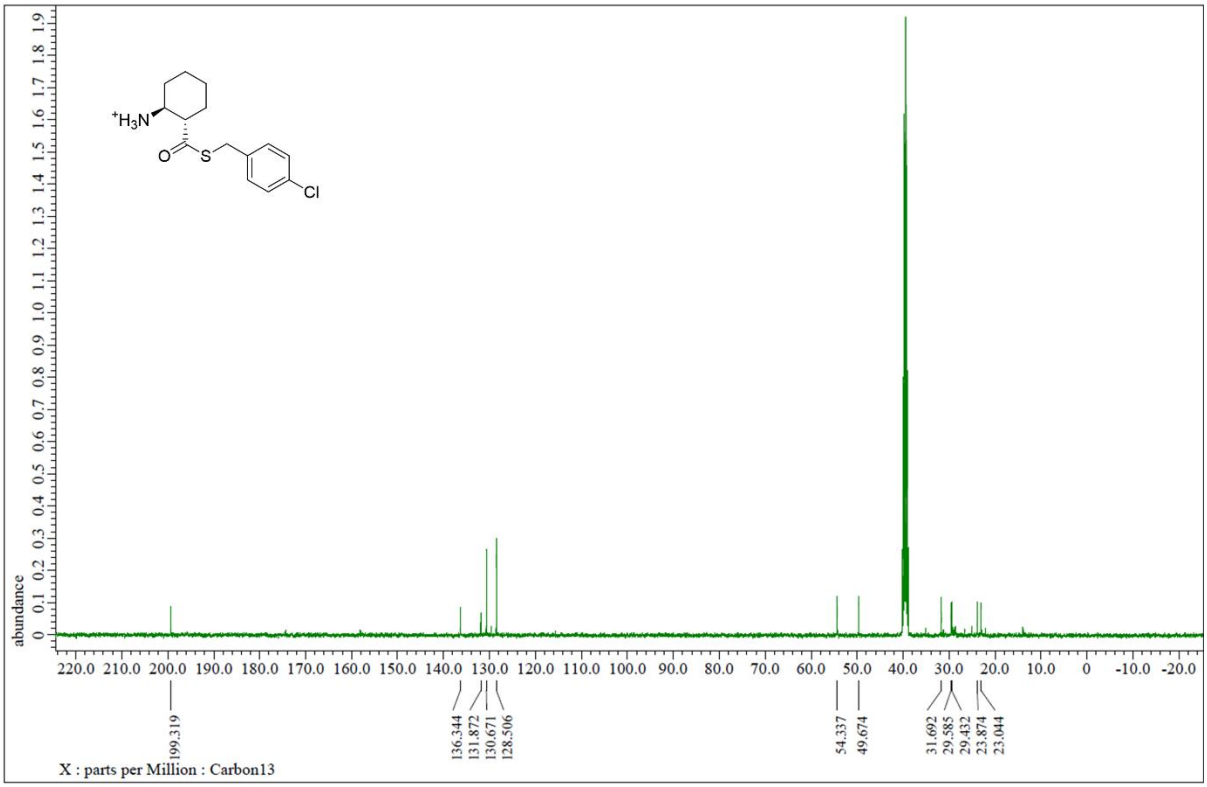


^{13}C NMR (101 MHz, $CDCl_3$) δ 200.1, 154.9, 136.4, 133.0, 130.2, 128.7, 79.4, 58.0, 51.6, 33.1, 32.4, 29.6, 28.4, 24.8, 24.6

Supplementary Fig. 56. ^{1}H NMR and ^{13}C NMR spectra of Boc-(1*S*,2*S*)-2-ACHC-CBT

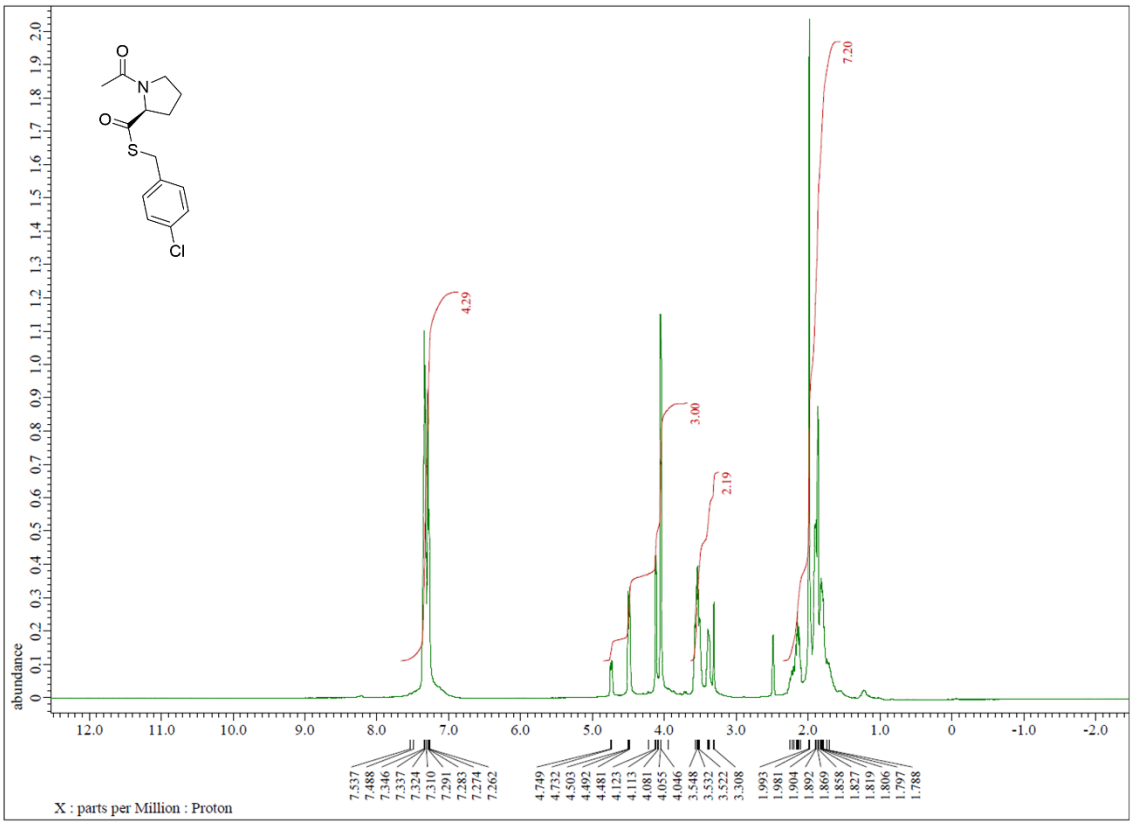


^{1}H NMR (400 MHz, DMSO-d_6) δ 8.04 (s, 3H), 7.41-7.30 (m, 4H), 4.23-4.11 (m, 2H), 3.30-3.18 (m, 1H), 2.78-2.72 (m, 1H), 2.02-1.92 (m, 2H), 1.70-1.60 (m, 2H), 1.37-1.22 (m, 4H)

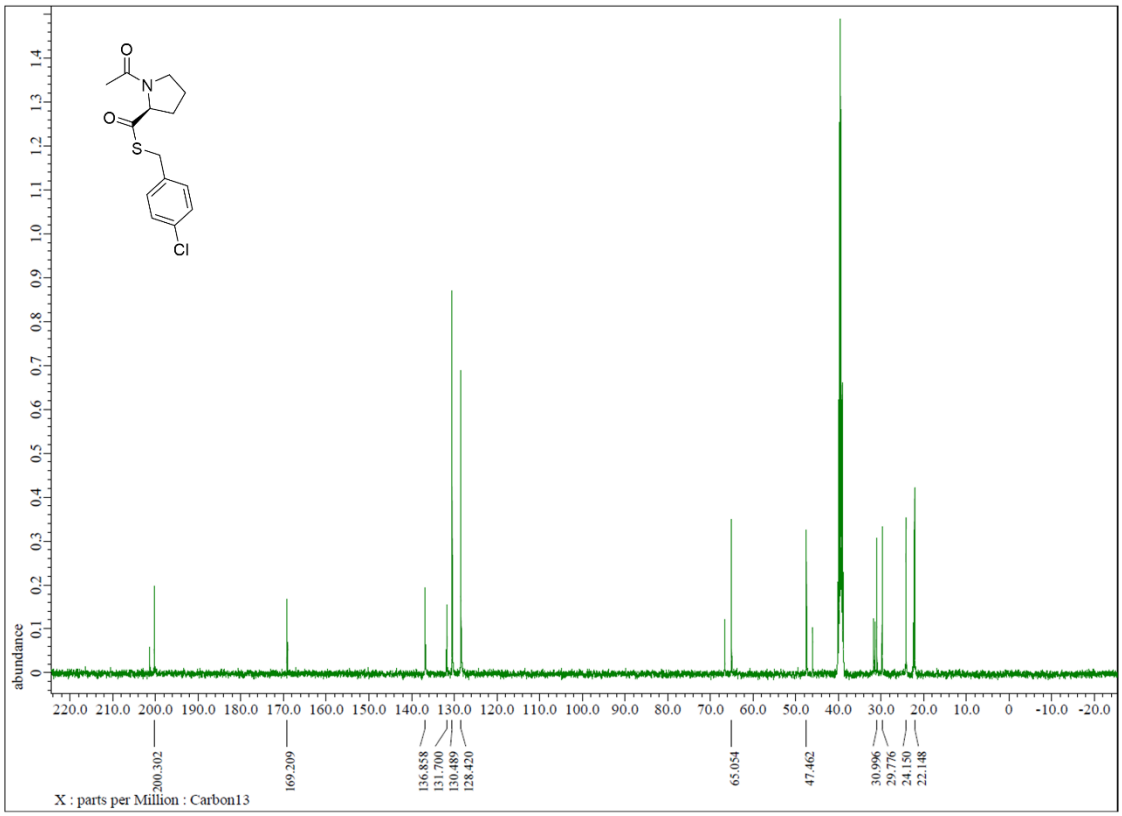


^{13}C NMR (101 MHz, DMSO-d_6) δ 199.3, 136.3, 131.9, 130.7, 128.5, 54.3, 49.7, 31.7, 29.6, 29.4, 23.9, 23.0

Supplementary Fig. 57. ^{1}H NMR and ^{13}C NMR spectra of (1*S*,2*S*)-2-ACHC-CBT

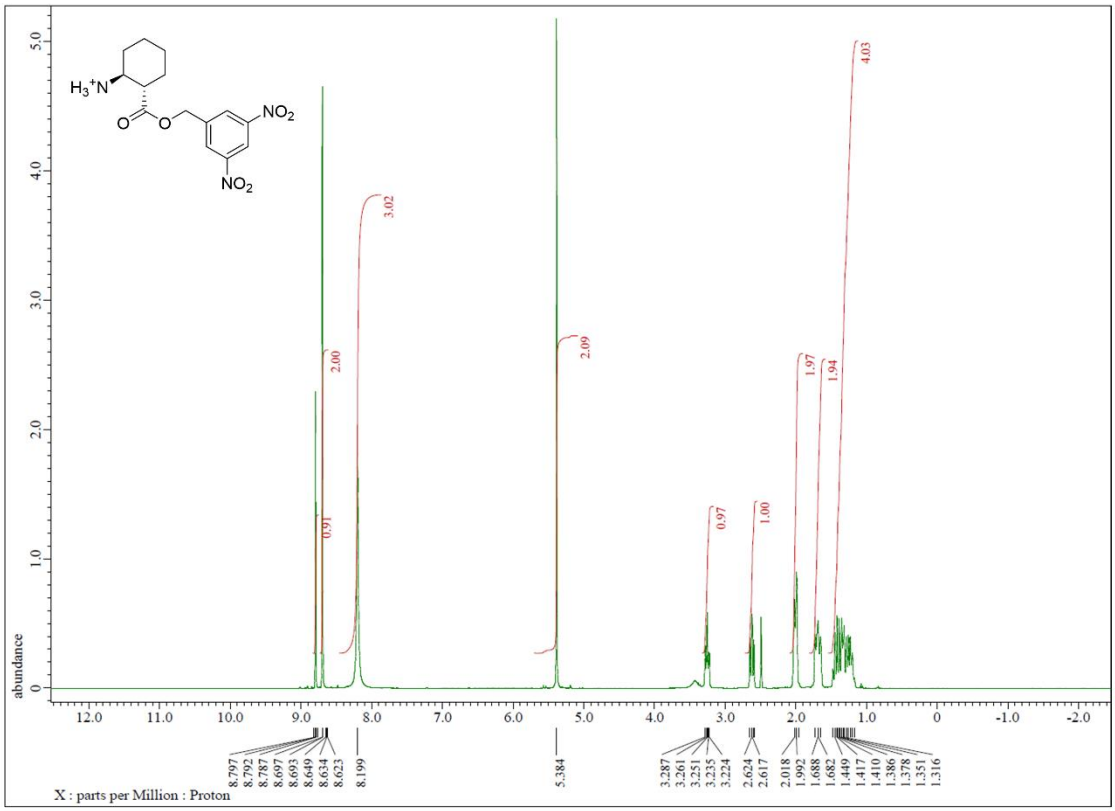


^{1}H NMR (400 MHz, DMSO-d_6) δ 7.54-7.26 (m, 4H), 4.75-3.94 (m, 3H), 3.57-3.31 (m, 2H), 2.25-1.71 (m, 7H)

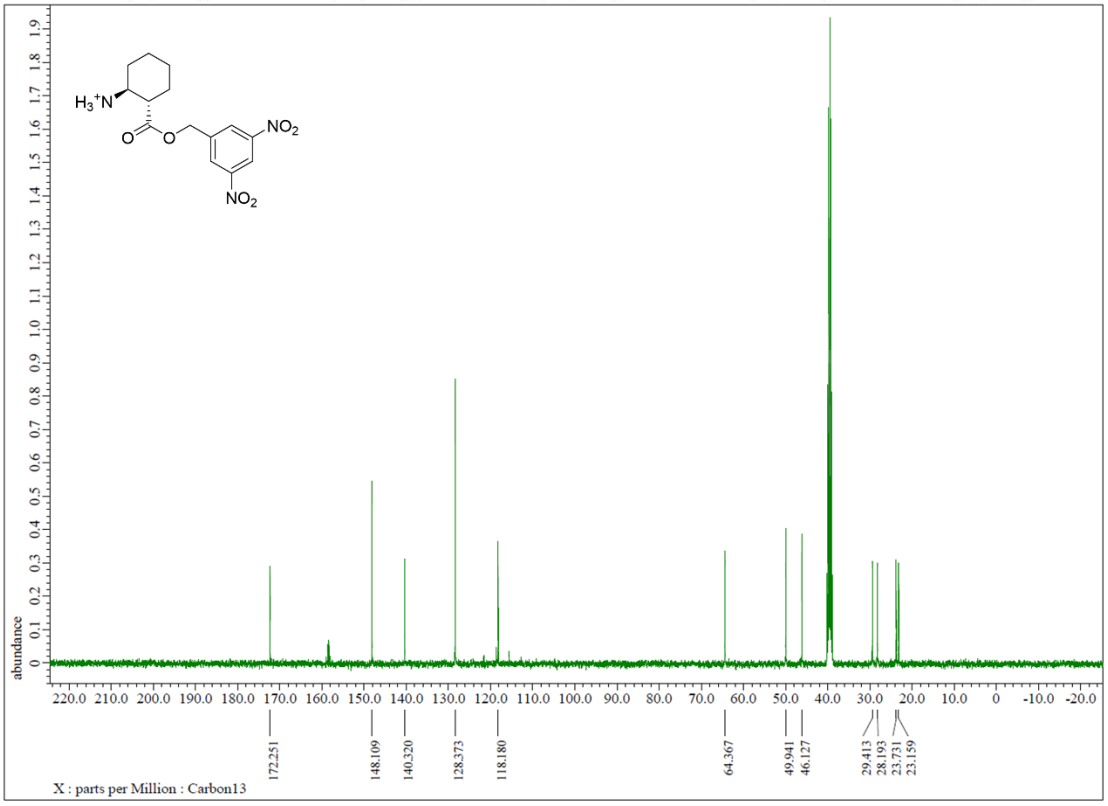


^{13}C NMR (101 MHz, DMSO-d_6) δ 200.3, 169.2, 136.9, 131.7, 130.5, 128.4, 65.1, 47.5, 31.0, 29.8, 24.2, 22.1

Supplementary Fig. 58. ^{1}H NMR and ^{13}C NMR spectra of Ac-L-Pro-CBT

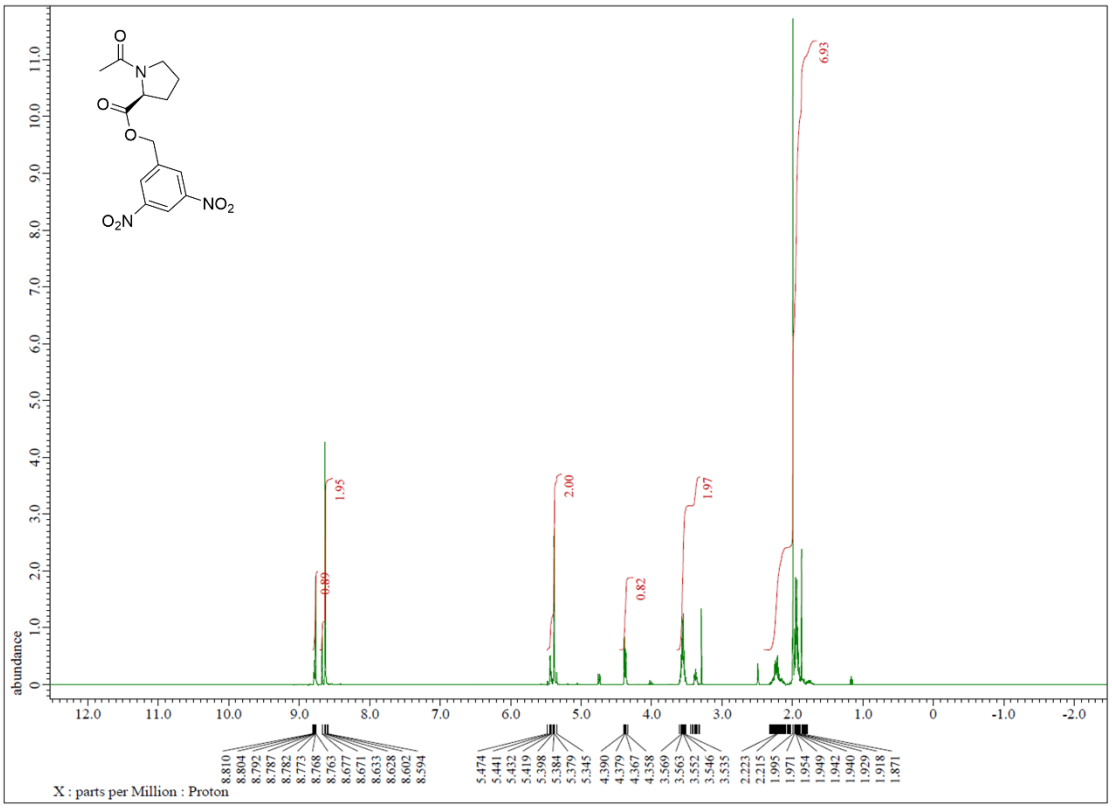


^{1}H NMR (400 MHz, DMSO-d_6) δ 8.82-8.76 (m, 1H), 8.70-8.62 (m, 2H), 8.20 (s, 3H), 5.38 (s, 2H), 3.29-3.22 (m, 1H), 2.65-2.59 (m, 1H), 2.02-1.95 (m, 2H), 1.73-1.65 (m, 2H), 1.48-1.17 (m, 4H)

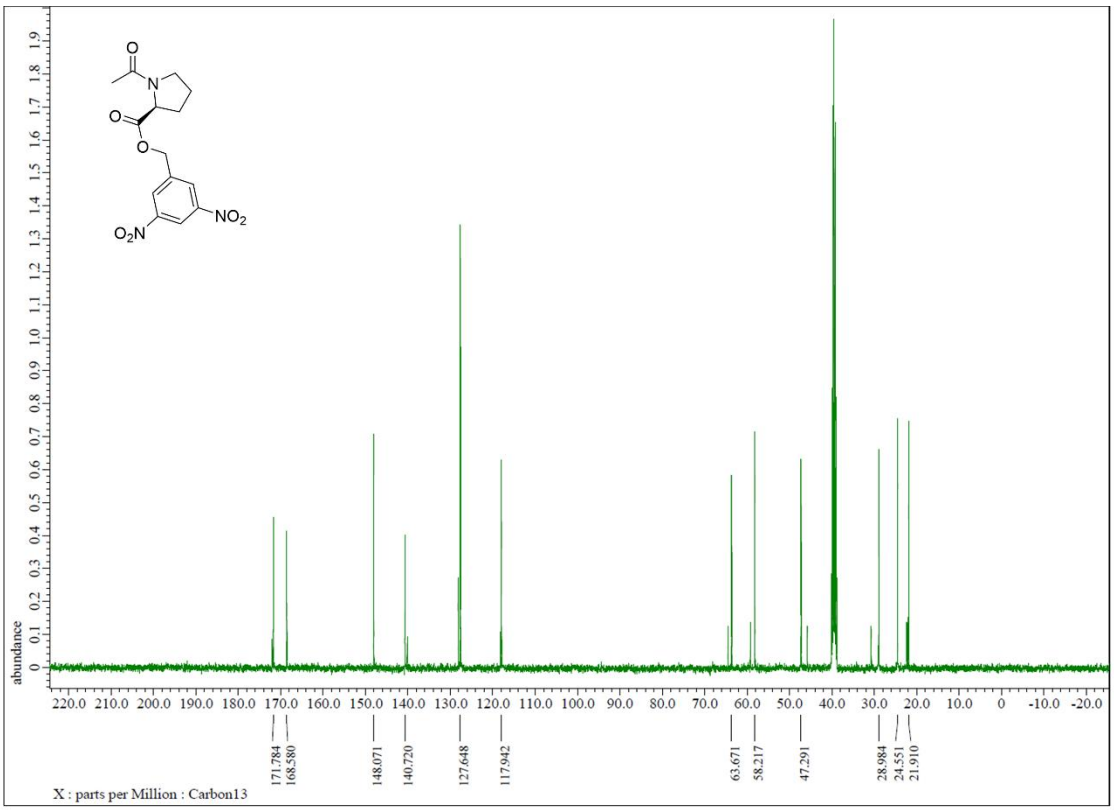


^{13}C NMR (101 MHz, DMSO-d_6) δ 172.3, 148.1, 140.3, 128.4, 118.2, 64.4, 49.9, 46.1, 29.4, 28.2, 23.7, 23.2

Supplementary Fig. 59. ^{1}H NMR and ^{13}C NMR spectra of (1*S*,2*S*)-2-ACHC-DBE

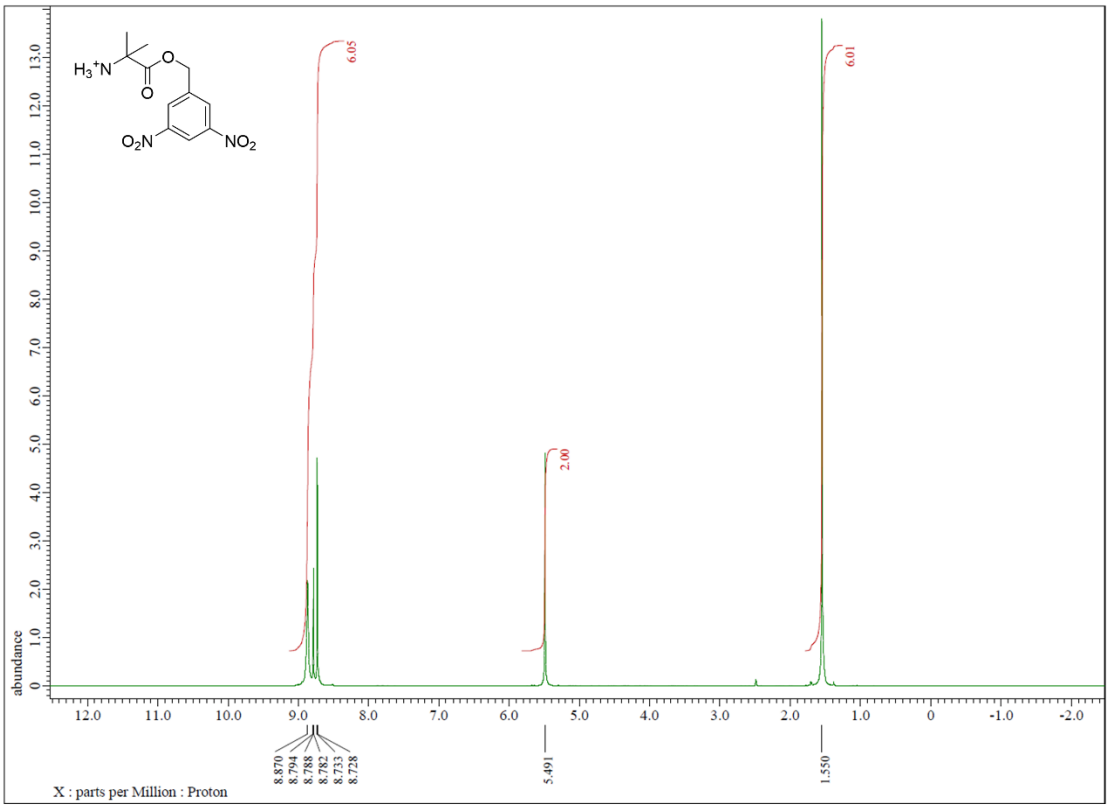


^{1}H NMR (400 MHz, DMSO-d_6) δ 8.80-8.76 (m, 1H), 8.68-8.59 (m, 2H), 5.47-5.34 (m, 2H), 4.39-4.34 (m, 1H), 3.60-3.32 (m, 2H), 2.33-1.78 (m, 7H)

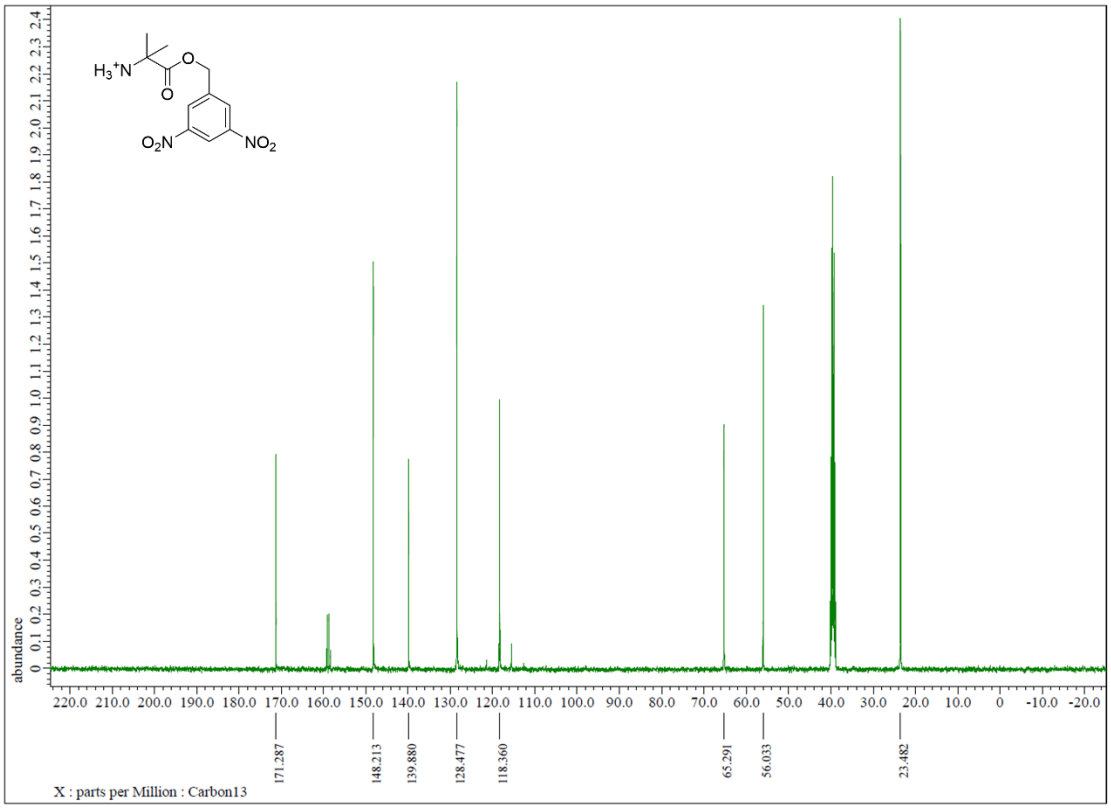


^{13}C NMR (101 MHz, DMSO-d_6) δ 171.8, 168.6, 148.1, 140.7, 127.6, 117.9, 63.7, 58.2, 47.3, 29.0, 24.6, 21.9

Supplementary Fig. 60. ^{1}H NMR and ^{13}C NMR spectra of Ac-L-Pro-DBE

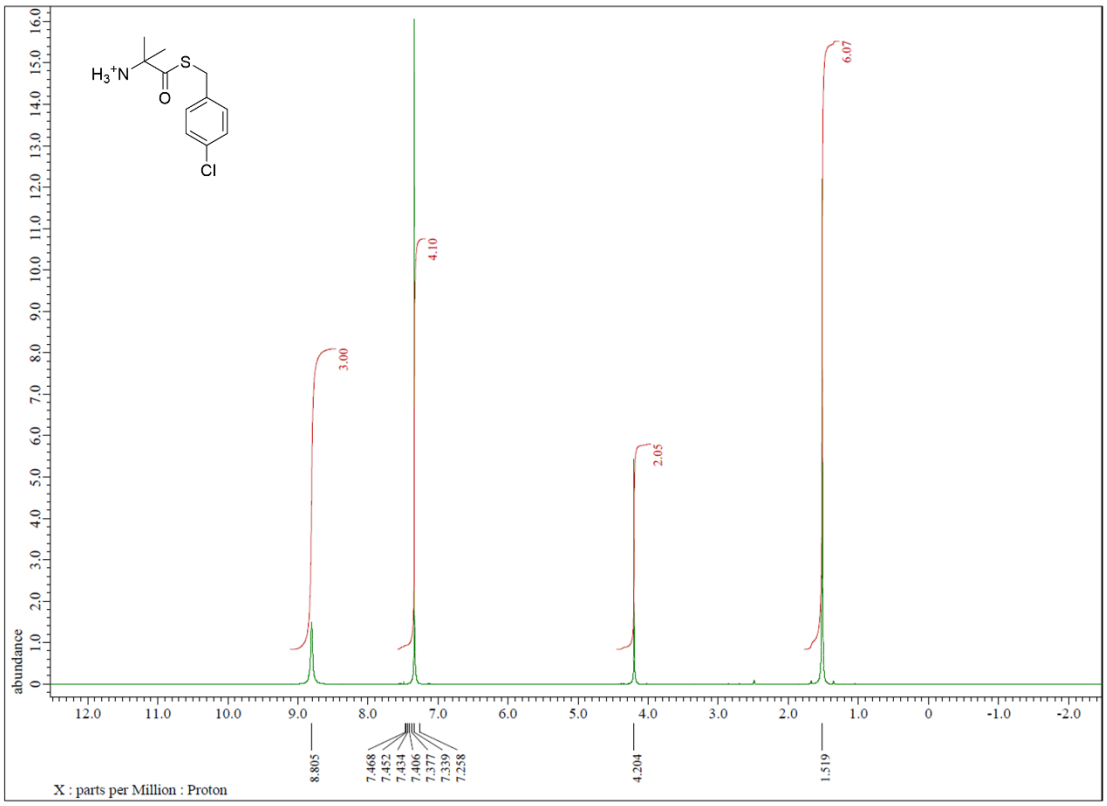


^{1}H NMR (400 MHz, DMSO-d_6) δ 8.87-8.73 (m, 6H), 5.49 (s, 2H), 1.55 (s, 6H)

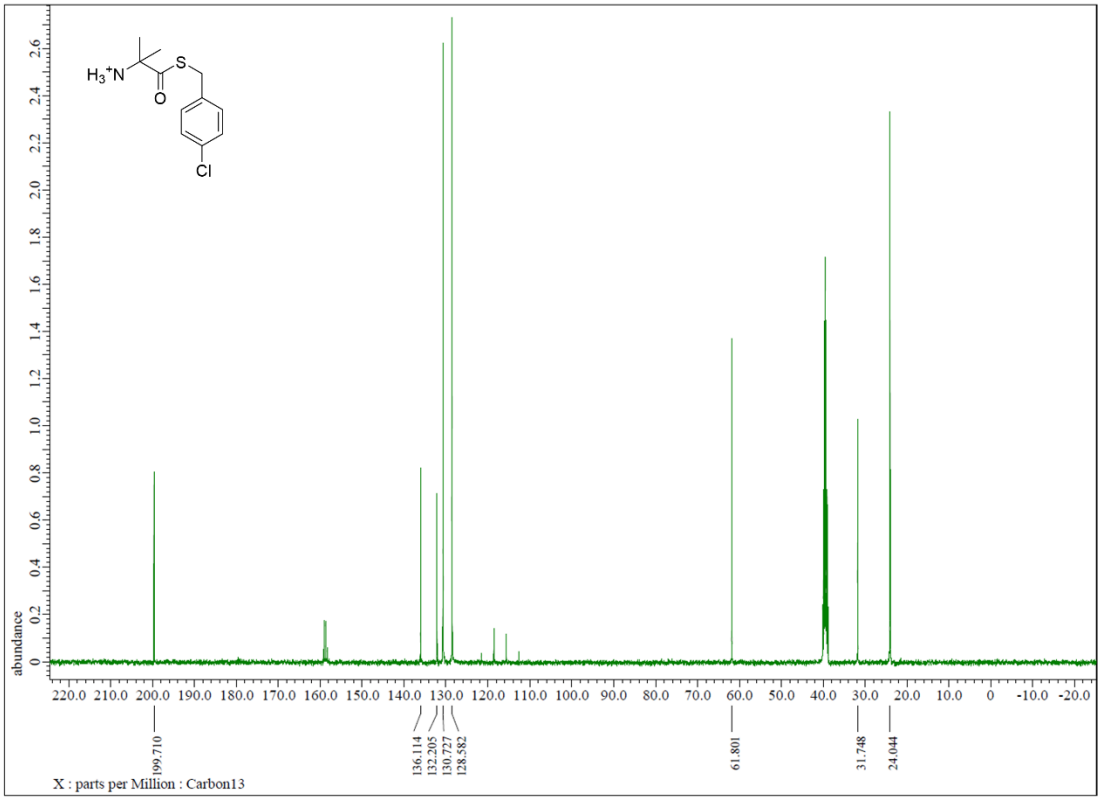


^{13}C NMR (101 MHz, DMSO-d_6) δ 171.3, 148.2, 139.9, 128.5, 118.4, 65.3, 56.0, 23.5

Supplementary Fig. 61. ^{1}H NMR and ^{13}C NMR spectra of Aib-DBE



^{1}H NMR (400 MHz, DMSO-d_6) δ 8.81 (s, 3H), 7.47-7.26 (m, 4H), 4.20 (s, 2H), 1.52 (s, 6H)



^{13}C NMR (101 MHz, DMSO-d_6) δ 199.7, 136.1, 132.2, 130.7, 128.6, 61.8, 31.7, 24.0

Supplementary Fig. 62. ^{1}H NMR and ^{13}C NMR spectra of Aib-CBT

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