Modular polyketide synthases and *cis*-double bond formation: Establishment of activated *cis*-3-cyclohexylpropenoic acid as the diketide intermediate in phoslactomycin biosynthesis

Supporting Information

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Materials and Methods

General methods. All reactions were carried out under nitrogen atmosphere using dry solvents under anhydrous conditions, unless otherwise noted. The solvents used were ACS grade from Fisher. Reagents were purchased from Aldrich and Acros, and used without further purification. Triethyl phosphonoacetate-2-^{13}C was purchased from Aldrich. Reactions were monitored by thin-layer chromatography (TLC) carried out on 0.20 mm POLYGRAM® SIL silica gel plates (Art.-Nr. 805 023) with fluorescent indicator UV_{254} using UV light as a visualizing agent. Normal phase flash column chromatography was carried out using Davisil® silica gel (100-200 mesh, Fisher). Preparative thin-layer chromatography (PTLC) separations were carried out on 1 mm, or 2 mm E. Merck silica gel plates (60F-254). ^{1}H NMR spectra were recorded on Varian INOVA 600 and Nicolet NM-500 MHz (modified with a Tecmag Libra interface) instruments calibrated using residual undeuterated solvent as an internal reference. ^{13}C NMR spectra were recorded on a Bruker AMX-400 NMR spectrometer. Coupling constants (*J*) were expressed in Hertz. The following abbreviations were used to explain the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. High-resolution mass spectra (HRMS) were recorded on a Micromass LCT Electrospray mass spectrometer performed at the Mass Spectrometry & Proteomics Facility (The Ohio State University).

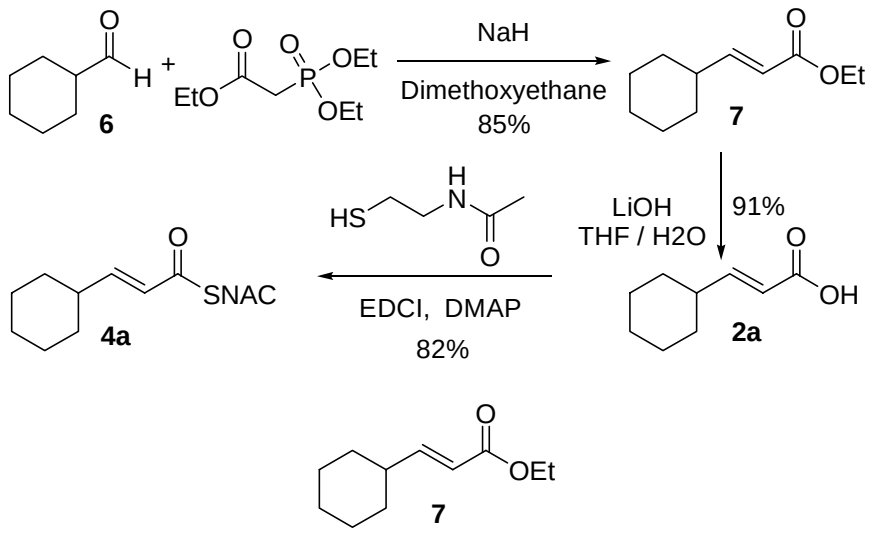
Construction of Δ*chcA* and Δ*plm1* mutants of *Streptomyces* sp. HK 803. An NP2 mutant was generated with an 81bp in-frame "scar" sequence on the chromosome of NP1 mutant[1] (*ΔplmS*$_2$ of *Streptomyces* sp. HK 803 that produces only PLM B) by replacing the *aac (3)IV* resistance marker and *oriT* using a PCR-targeting based gene replacement method.[2] The production of

PLM B in the NP2 mutant strain was confirmed and this mutant was used in *chcA* and *plm1* genes replacement studies.

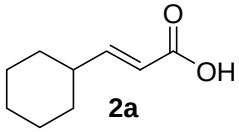
The Δ*chcA* mutant was created from NP2 by replacing *chcA* with an *aac (3)IV* resistance marker and *oriT* cassette. The replacement was first accomplished in cosmid 3A11 and subsequently in the chromosome of the NP2 mutant using approaches previously described.[1] For the gene replacement, the forward primer: 5'-GCGGCAGGCCGGGACCGTCACCGCGGGAGGTGCGCGATG***ATTCCGGGGATCCGTCG ACC*** 3', and reverse primer: 5'-AAGACATGGTCGTACACGTCGCACGCCCCTCGTCTCTCA***TGTAGGCTGGAGCTGCTT C*** 3' were used (sequence homologous to pIJ773 disruption cassette is shown in bold, italicized text). Allelic replacement of the *chcA* gene with the *aac (3)IV* resistance marker and *oriT* cassette in the chromosome of the resulting NP3 mutant was confirmed by PCR amplification and sequencing.

The *plm1* gene of PLM gene cluster was replaced with an 81bp in frame "scar" sequence on the chromosome of NP2 following standard PCR-targeting methodology [2] and cosmid 10B4. [1] The forward primer: 5'-CCCGCGCTGGTCGTCGTCCACGACGAGACCGGCGGCGTG***ATTCCGGGGATCCGTCGA CC*** 3' and reverse primer: 5'-GGCGGCCCCGCCCGGGGCCGGCGGGGCCGCCCGGCGTCA***TGTAGGCTGGAGCTGCT TC*** 3' were used (sequence homologous to pIJ773 disruption cassette is shown in bold, italicized text). The correct allelic replacement of the *plm1* gene with the 81bp scar sequence of cassette pIJ773 in the chromosome of the resulting NP9 mutant was confirmed by PCR amplification and sequencing..

Synthesis of compounds 2 and 4:

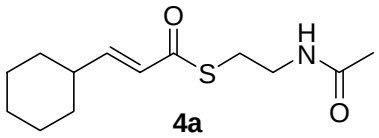


(*E*)-ethyl 3-cyclohexylpropenoate (7). Triethyl phosphonoacetate (4.8 g, 21.4 mmol) was added dropwise to a cooled (0 ^{o}C) suspension of NaH (470 mg, 19.6 mmol) in dimethoxyethane (30 ml). The reaction mixture was stirred at 0 ^{o}C for 30 min and then allowed to warm to room temperature. Cyclohexanecarboxaldehyde (**6**) (2.02 g, 18 mmol) was added and the mixture was stirred for additional 4 hr. The reaction was quenched with water (30 ml) and extracted with EtOAc (3 x 30 ml). The organic extracts were dried over anhydrous sodium sulfate and concentrated in vacuo. The crude product (diastereoselectivity, >15:1 *E*:*Z*) was subjected to flash column chromatography (silica gel, 10% EtOAC/hexanes) to give of 2.79 g (85%) of pure *E*-product **7**. ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.92 (dd, *J* = 15.9, 7.2 Hz, 1H), 5.76 (d, *J* = 15.9 Hz, 1H), 4.18 (q, *J* = 7.2 Hz, 2H), 2.14 (m, 1H), 1.78-1.74 (m, 5H), 1.29 (t *J* = 7.2 Hz, 3H), 1.23-1.14 (m, 5H).



(*E*)-3-cyclohexylpropenoic acid (2a). To a suspension of **7** (2 g, 10.99 mmol) in a mixture of THF (50 ml) and water (60 ml) was added LiOH.H_2O (1.39 g, 33.1 mmol). The reaction mixture

was stirred at 60 ^{o}C for 12 hr after which it was cooled to 0 ^{o}C and carefully acidified to pH 2-3 with 1N aqueous HCl. The mixture was extracted with EtOAc (3 x 30 ml) and the combined organic extracts were dried over anhydrous sodium sulfate and concentrated in vacuo. The crude product was subjected to flash column chromatography (silica gel, 10% EtOAc/hexanes) to give 1.54 g (91%) of **2a** as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.04 (dd, *J* = 15.9, 6.6 Hz, 1H), 5.78 (dd, *J* = 15.9, 1.5 Hz, 1H), 2.17 (m, 1H), 1.79-1.68 (m, 5H), 1.34-1.13 (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.49, 157.16, 118.31, 40.56, 31.60, 25.95, 25.71; HRMS calcd for $C_9H_{14}O_2 + Na^+$ 177.0891; found 177.0897 [M + Na^+].

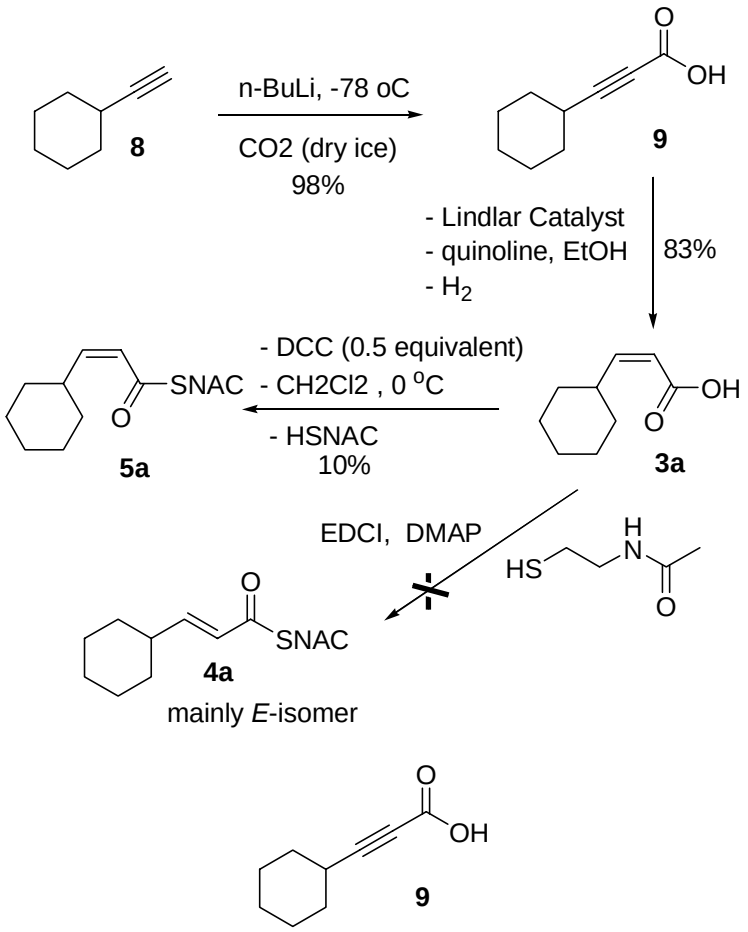


4a

(*E*)-S-2-acetamidoethyl 3-cyclohexylprop-2-enethioate (4a). A solution of carboxylic acid **2a** (50 mg, 0.325 mmol) in anhydrous methylene chloride (2 ml) was cooled to 0 ^{o}C for 15 min. To this solution was added N-acetyl cysteamine (47 mg, 0.394 mmol) followed by 4-(N,N-dimethylamino)pyridine (10 mg, 0.082 mmol), and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (75.5 mg, 0.394 mmol). The mixture was allowed to warm to room temperature and stirred overnight. Saturated aqueous NH_4Cl solution (2 ml) was added and the organic phase was separated. The aqueous phase was extracted with 3 x 3 ml of ether and the combined organic phases were dried over anhydrous sodium sulfate and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, 30% EtOAc/hexanes) to give 68 mg (82%) of **4a** as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.90 (dd, *J* = 15.9, 6.9 Hz, 1H), 6.10 (dd, *J* = 15.9, 1.5 Hz, 1H), 5.9 (m, 1H), 3.47 (q, *J* = 6, 2H), 3.10 (t, *J* = 6, 2H), 2.17 (m, 1H), 1.97 (s, 3H), 1.79-1.68 (m, 5H), 1.34-1.13 (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 190.2, 169.9,

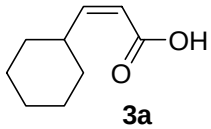
150.9, 125.5, 40.0, 39.4, 31.1, 27.8, 25.4, 25.2, 22.8; HRMS calcd for $C_{13}H_{21}NO_2S + Na^+$ 278.1191; found 278.1187 [$M + Na^+$].

Synthesis of compounds 3a and 5a:



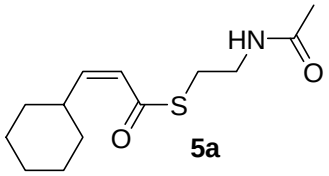
3-cyclohexylpropiolic acid (9). To a solution of alkyne **8** (1 g, 9.25 mmol) in Et_2O (40 ml) at -78 ^oC, was added a solution of n-BuLi (6.25 ml, 10 mmol of 1.6 M in hexane) dropwise. After stirring for 1 hr at -78 ^oC, excess solid carbon dioxide (dry ice) was added and the reaction mixture was allowed to warm to room temperature. The reaction was quenched with 0.5 M aqueous citric acid (40 ml) and the aqueous layer was extracted with EtOAc (3 x 30 ml). The combined organic extracts were dried over anhydrous sodium sulfate, concentrated *in vacuo*, and the residue was purified by column chromatography (silica gel, 10% EtOAc/hexanes) to give

1.38 g (98%) of **9** as a yellow solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 10.0 (br, 1H), 2.54-2.42 (m, 1H), 1.83-1.72 (m, 2H), 1.72-58 (m, 2H), 1.54-1.40 (m, 3H), 1.38-1.20 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 159.2, 96.7, 72.1, 31.0, 28.2, 24.5, 24.1.



3a

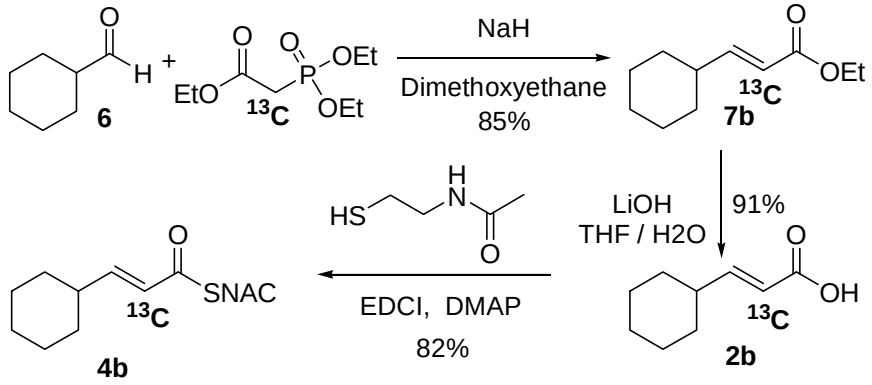
(*Z*)-3-cyclohexylpropenoic acid (3a). A mixture of Lindlar catalyst (51 mg, 5 wt% of Pd on $CaCO_3$, poisoned with lead) and quinoline (42 µL, 0.355 mmol) in anhydrous ethanol (20 ml) was stirred at room temperature for 20 min. A solution of compound **9** (500 mg, 3.29 mmol) in ethanol (20 ml) was added and the mixture was stirred for an additional 20 min at room temperature. The reaction mixture was placed under hydrogen atmosphere (balloon) and stirred for additional 4 hr. The catalyst was removed by filtration through celite and the filtrate was concentrated *in vacuo*. The crude product was subjected to flash column chromatography (silica gel, 10% EtOAc/hexanes) to give 420 mg (83%) of **3a** as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 12.18 (br, 1H), 6.16 (dd, *J* = 11.4, 9.9 Hz, 1H), 5.68 (dd, *J* = 11.4, 1 Hz, 1H), 3.32-3.29 (m, 1H), 1.74-1.68 (m, 5H), 1.39-1.09 (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.8, 157.8, 116.7, 32.0, 31.8, 25.4, 24.9; HRMS calcd for $C_9H_{14}O_2$ + Na^+ 177.0891; found 177.0890 [M + Na^+].



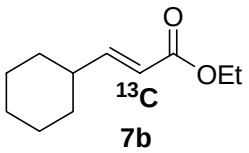
5a

(*Z*)-S-2-acetamidoethyl 3-cyclohexylprop-2-enethioate (5a).* To a cooled (0 ^{o}C) solution of acid **3a** (105 mg, 0.682 mmol) in anhydrous CH_2Cl_2 (5 ml) was added dicyclohexylcarbodiimide (DCC) (70 mg, 0.34 mmol) and mixture was stirred at 0 ^{o}C for 30 min. N-acetyl cysteamine (97 mg, 0.818 mmol) was added and the reaction mixture was stirred at 0 ^{o}C for additional 2 hr after which saturated aqueous NH_4Cl solution (2 ml) was added and the organic phase was separated. The aqueous phase was extracted with 3 x 3 ml of ether and the combined organic phases were dried over anhydrous sodium sulfate and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, 30% EtOAc/hexanes) to give 17 mg (10%) of **5a** as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.97 (d, J = 11.1 Hz, 1H), 5.91-5.83 (m, 2H), 3.47 (q, J = 6, 2H), 3.23-3.18 (m, 1H), 3.08 (t, J = 6, 2H), 1.98 (s, 3H), 1.74-1.67 (m, 5H), 1.34-1.08 (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 189.8, 170.2, 153.1, 124.0, 39.8, 38.4, 32.3, 28.6, 25.8, 25.4, 23.2; HRMS calcd for $C_{13}H_{21}NO_2S + Na^+$ 278.1191; found 278.1190 [M + Na^+].

Synthesis of 2-^{13}C labeled compounds 2b and 4b:

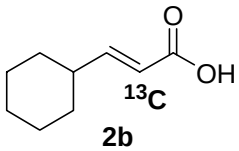


* Performing the thioesterification reaction using DMAP and, either DCC or EDCI, resulted in mainly the isomerized *E*-product. Various other methods failed to give the desired *Z*-product.



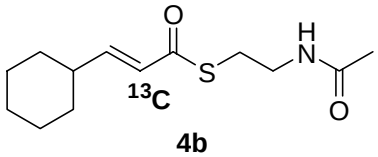
7b

(*E*)-ethyl [2-^{13}C]-3-cyclohexylpropenoate (7b). Synthesized as described above for compound **7**, except using triethyl phosphonoacetate-2-^{13}C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.92 (ddd, *J* = 16, 7, 2 Hz, 1H), 5.76 (ddd, *J* = 162.5, 16, 1.5 Hz, 1H), 4.19 (q, *J* = 7 Hz, 2H), 2.14 (m, 1H), 1.78-174 (m, 5H), 1.3 (t *J* = 7 Hz, 3H), 1.23-1.14 (m, 5H).



2b

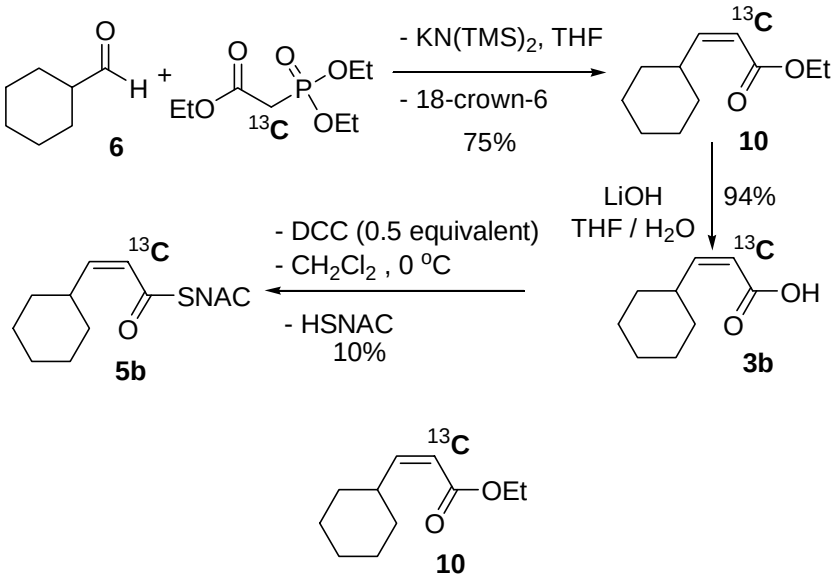
(*E*)-[2-^{13}C]-3-cyclohexylpropenoic acid (2b). Synthesized as described above for compound **2a**. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.04 (ddd, *J* = 16, 7, 2 Hz, 1H), 5.78 (ddd, *J* = 163, 16, 1.5 Hz, 1H), 2.17 (m, 1H), 1.78-174 (m, 5H), 1.34-1.13 (m, 5H); HRMS calcd for $C_8{}^{13}CH_{14}O_2 + Na^+$ 178.0925; found 178.0931 [$M + Na^+$].



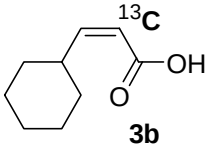
4b

(*E*)-[2-^{13}C]-S-2-acetamidoethyl 3-cyclohexylprop-2-enethioate (4b). Synthesized as described above for compound **4a**. ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.88 (ddd, *J* = 15.5, 6.5, 15.5 Hz, 1H), 6.08 (ddd, *J* = 162, 16, 1.5 Hz, 1H), 5.91-5.89 (m, 1H), 3.47 (q, *J* = 6, 2H), 3.1 (t, *J* = 6, 2H), 2.15 (m, 1H), 1.97 (s, 3H), 1.79-175 (m, 4H), 1.71-1.68 (m, 1H), 1.34-1.13 (m, 5H); HRMS calcd for $C_{12}{}^{13}CH_{21}NO_2S + Na^+$ 279.1224; found 279.1227 [$M + Na^+$].

Synthesis of 2-^{13}C labeled compounds 3b and 5b:

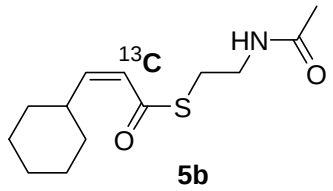


(*Z*)-ethyl [2-^{13}C]-3-cyclohexylpropenoate (10).[3] A solution of 18-crown-6 (1.32 g, 5 mmol) and triethyl phosphonoacetate-2-^{13}C (226 mg, 1 mmol) in anhydrous THF (20 ml) was cooled to -78 ^{o}C. $KN(TMS)_2$ (2 ml of 0.5 M in toluene, 1 mmol) was added dropwise and the reaction mixture was stirred for 5 min after which aldehyde **6** (112 mg, 1 mmol) was added. The resulting mixture was stirred at -78 ^{o}C for additional 30 min and then quenched with saturated aqueous NH_4Cl solution (20 ml). The product was extracted with 3 x 20 ml of EtOAc and the organic extracts were dried over anhydrous sodium sulfate and concentrated *in vacuo* to give the crude product **10** as a mixture of *Z* and *E* isomers (*Z*:*E*, 10:1). Purification by flash column chromatography (silica gel, 10% CH_2Cl_2/hexanes) gave 136 mg (75%) of the pure *Z*-product **10**. ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.03 (dd, *J* = 11.5, 10 Hz, 1H), 5.66 (dd, *J* = 161.8, 11.5 Hz, 1H), 4.17 (q, *J* = 7 Hz, 2H), 3.33-3.26 (m, 1H), 1.75-171 (m, 5H), 1.29 (t *J* = 7 Hz, 3H), 1.26-1.10 (m, 5H).



(*Z*)-[2-^{13}C]-3-cyclohexylpropenoic acid (3b). Synthesized as described above for compound **2a**. ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.16 (dd, *J* = 11.5, 10 Hz, 1H), 5.69 (dd, *J* = 163, 11.5 Hz, 1H),

3.34-3.27 (m, 1H), 174-1.67 (m, 5H), 1.37-1.10 (m, 5H); HRMS calcd for $C_8{}^{13}CH_{14}O_2 + Na^+$ 178.0925; found 178.0927 [M + Na^+].



5b

(*Z*)-[2-^{13}C]-S-2-acetamidoethyl 3-cyclohexylprop-2-enethioate (5b). Synthesized as described above for compound **5a**. ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.97 (dd, *J* = 162.5, 11.5 Hz, 1H), 5.93-5.80 (m, 2H), 3.47 (q, *J* = 6, 2H), 3.23-3.16 (m, 1H), 3.08 (t, *J* = 6, 2H), 2.15 (m, 1H), 1.97 (s, 3H), 1.75-169 (m, 5H), 1.37-1.10 (m, 5H); HRMS calcd for $C_{12}{}^{13}CH_{21}NO_2S + Na^+$ 279.1224; found 279.1219 [M + Na^+].

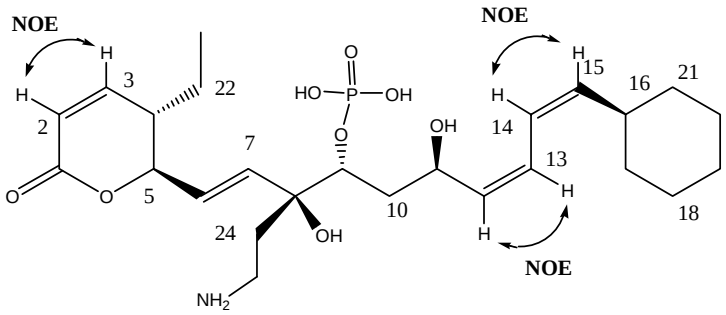
Production, isolation and analysis of PLM B. The NP3 and NP9 mutants were maintained and grown as previously described.[1] Restoration of PLM B by addition of the respective precursors (dissolved in ethanol) in both mutants was studied in both solid and liquid cultures with consistent results. The production of PLM B in solid culture was analyzed by growing on a Petri dish containing 25 mL SY agar (soluble starch 1.0%, yeast extract 0.1%, N-Z amine type A 0.1%, agar 1.5%, pH was adjusted to 7.0) supplemented with 2 mg of the respective precursors (CHC, **2a**-**5a**) at 30 ^{o}C for 7 days. The solid media was then homogenized and extracted with 100 ml of methanol. The methanol extract was filtered and evaporated to dryness and finally the residue was dissolved in 2 mL of methanol: water (1:1 v/v). This crude extract solution was subjected to HPLC and LC-MS analysis. For the liquid culture, a loop full of spores of NP3 or NP9 mutant strain was inoculated in 50 ml seed medium (soluble starch 3.0%, dried yeast 1.0%, NaCl 0.3%, $CaCO_3$ 0.3%, pH 7.0) in a 500 mL baffled flask and incubated for 48 h at 28 ^{o}C with shaking at 170 rpm in dark. About 200 µL of the seed culture was inoculated in 5 ml of production medium

(soy bean meal 1.5%, dried yeast 0.2%, soluble starch 2.5%, meat extract 0.1%, NaCl 0.2%, $CaCO_3$ 0.4%, pH was adjusted to 7.2 before autoclaving) in 70 ml Erlenmeyer flask and incubated for 96 h at 28 ^{o}C with shaking at 170 rpm in dark. The non-labeled (CHC, **2a-5a**) and labeled (**2b-5b**) precursors (total amount of 1.2 mg) were added in four equal portions at 0, 24, 48, and 72 h. At the end of fermentation period the cultures were harvested, the mycelia were removed by centrifugation, and the resulting supernatant was analyzed directly by HPLC and LC-MS. Relative levels of Plm B restoration were obtained through analyses of three separate fermentations of each mutant with each of the respective precursors.

HPLC and LC-MS analysis. A 500 µl of the filtered supernatant of the fermentation broth was analyzed by using an Agilent 1100 HPLC system connected to a diode array detector. The PLM B from the supernatant was analyzed with a 5 µm Discovery HS C18 reverse phase column (4.6 x 250 mm, Supelco, Belefonte, PA) using a linear gradient between solvent A (acetonitrile: water; 20:80 with 0.05% formic acid) and solvent B (acetonitrile: water; 80:20 with 0.05% formic acid) from 0% to 60% over 40 minutes at a flow rate of 1.0 ml/min and detected at 235 nm. The purification of PLM B from fermentation broth of NP3 mutant strain for NMR analysis was carried out as described previously.[4]

The LC-MS analysis was carried out using 100 µl of the supernatant under same solvent and gradient conditions with flow rate of 0.3 ml/min using a Surveyor HPLC system (ThermoFinnigan) connected to a diode array detector equipped with a 2.1 µm Discovery HS C18 reverse phase column (4.6 x 250 mm, Supelco). Mass spectra were collected on an LCQ quadrupole ion trap (ThermoFinnigan) mass spectrometer equipped with an electrospray ion source operating in positive mode.

Feeding of (*E*)-3-cyclohexylacrylic acid (2a) to Δ*chcA* and isolation of PLM B. Compound **2a** was fed to Δ*chcA* mutant and PLM B was isolated as described above. The purified PLM B co-eluted with a standard PLM B sample using the HPLC conditions described above. The ^{1}H NMR of purified PLM B was in good agreement with that of the natural prodcut.[5]



Purified PLM B (1). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.07 (dd, *J* = 9.9, 5.1 Hz, 1H), 6.25-6.20 (m, 2H), 6.04 (dd, *J* = 15.3, 6.9 Hz, 1H), 6.0 (d, *J* = 9.6 Hz, 1H), 5.94 (d, *J* = 15.6 Hz, 1H), 5.39 (dd, *J* = 9, 9 Hz, 1H), 5.31 (dd, *J* = 9, 9 Hz, 1H), 5.09 (dd, *J* = 5, 5 Hz, 1H), 4.92 (m, 1H), 4.27 (dd, *J* = 9.2, 9.2 Hz, 1H), 3.06-2.97 (m, 2H), 2.54 (m, 1H), 2.44 (m, 1H), 2.03-1.94 (m, 2H), 1.71-0.98 (m, 16H), 0.94 (t, *J* = 7 Hz, 3H); HRMS calcd for $C_{25}H_{40}NO_8P + Na^+$ 536.2389; found 536.2411 [$M + Na^+$].

NOESY of purified PLM B: The *cis* (*Z*) stereochemistry of the three double bonds (C_2-C_3, C_{12}-C_{13}, and C_{14}-C_{15}) was confirmed by NOESY experiment that showed three strong NOE interaction signals between the protons on the corresponding carbons.

References

(1) Palaniappan, N.; Kim, B. S.; Sekiyama, Y.; Osada, H.; Reynolds, K. A. *J Biol Chem* **2003**, *278*, 35552-35557.

(2) Gust, B.; Challis, G. L.; Fowler, K.; Kieser, T.; Chater, K. F. *Proc. Natl. Acad. Sci. U S A.* **2003**, *100*, 1541-1546.

(3) Still, W. C.; Gennari, C. *Tetrahedron Lett.* **1983**, *24*, 4405-4408.

(4) Choudhuri, S. D.; Ayers, S.; Soine, W. H.; Reynolds, K. A. *J Antibiot (Tokyo)* **2005**, *58*, 573-582.

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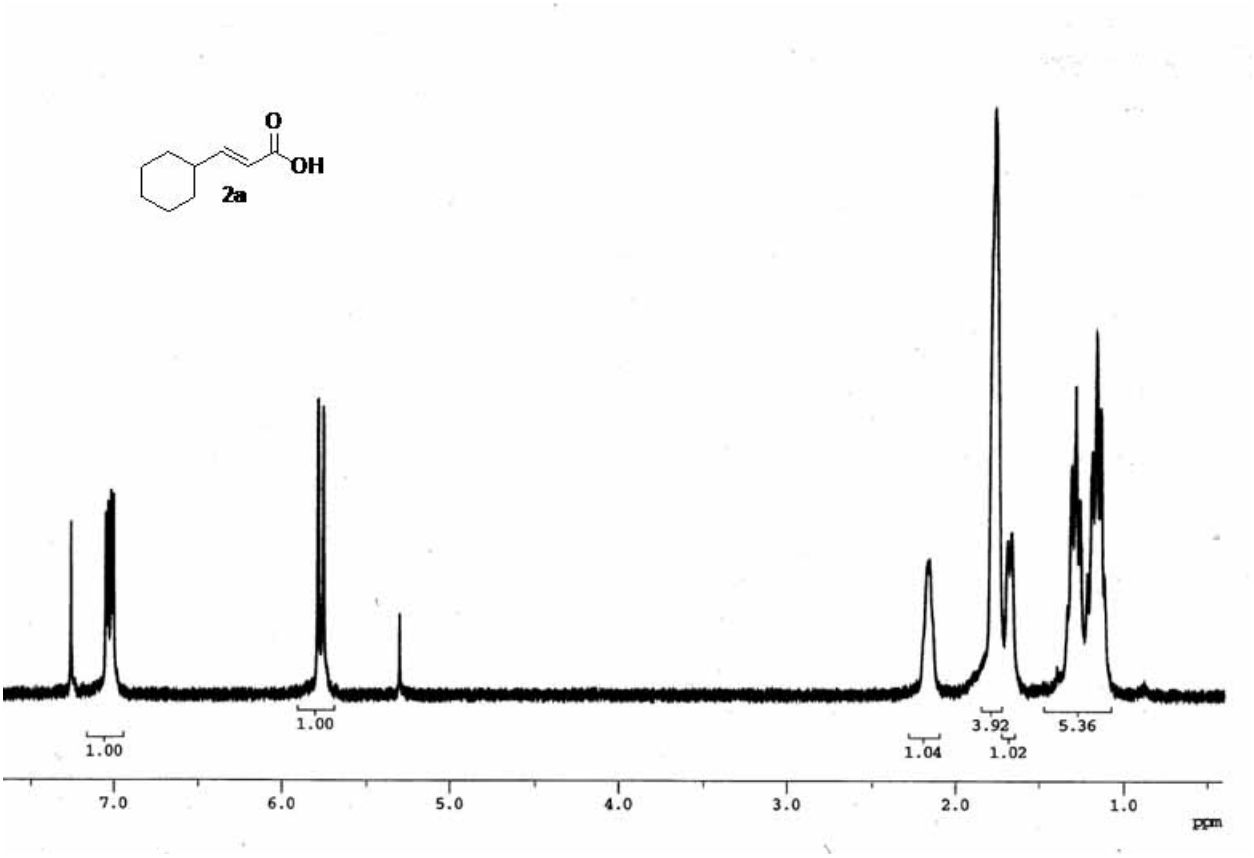
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OH
2a
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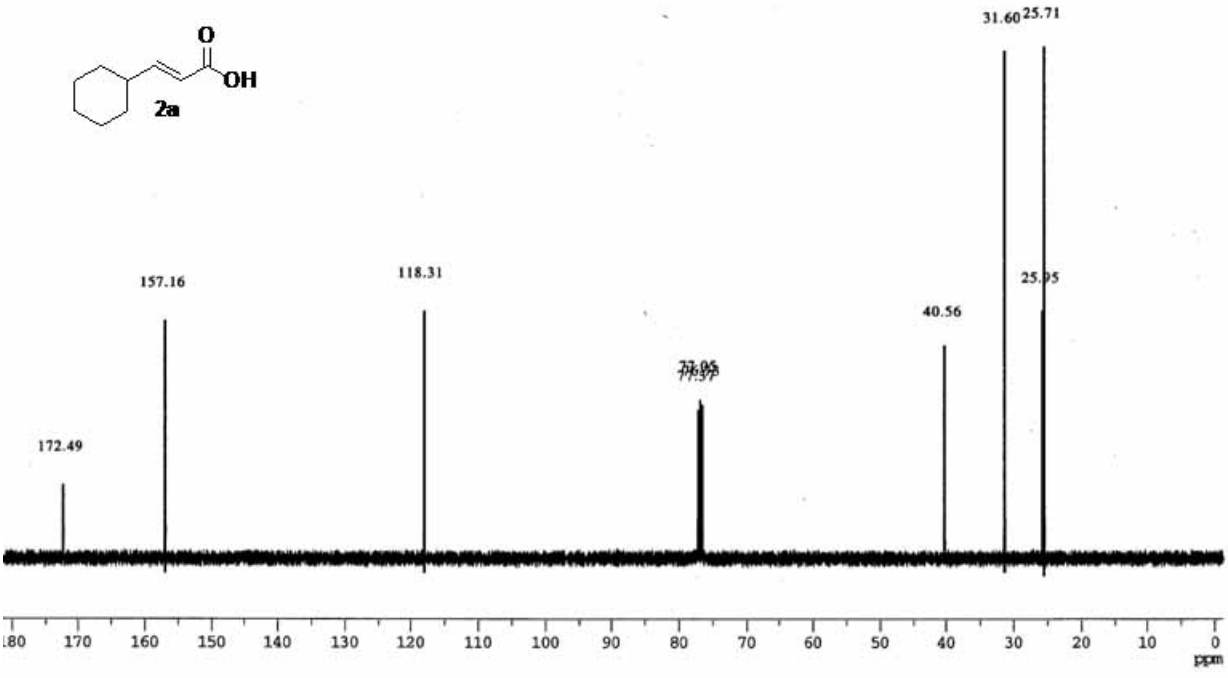
1.00
1.04
3.92
1.02
5.36
7.0
6.0
5.0
4.0
3.0
2.0
1.0
ppm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OH
2a
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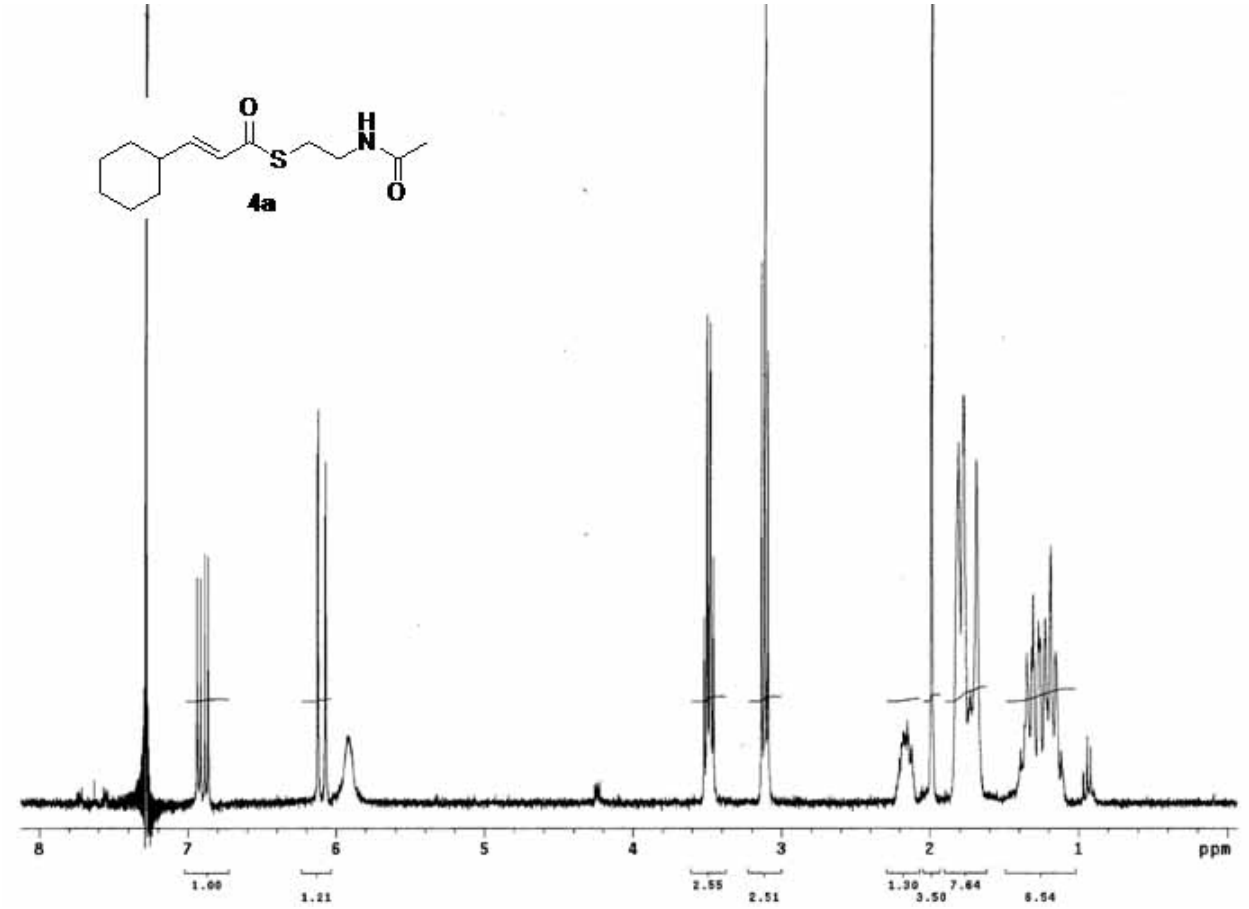
25.71
157.16
118.31
40.56
25.05
172.49
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
ppm


4a
8
7
6
5
4
3
2
1
ppm
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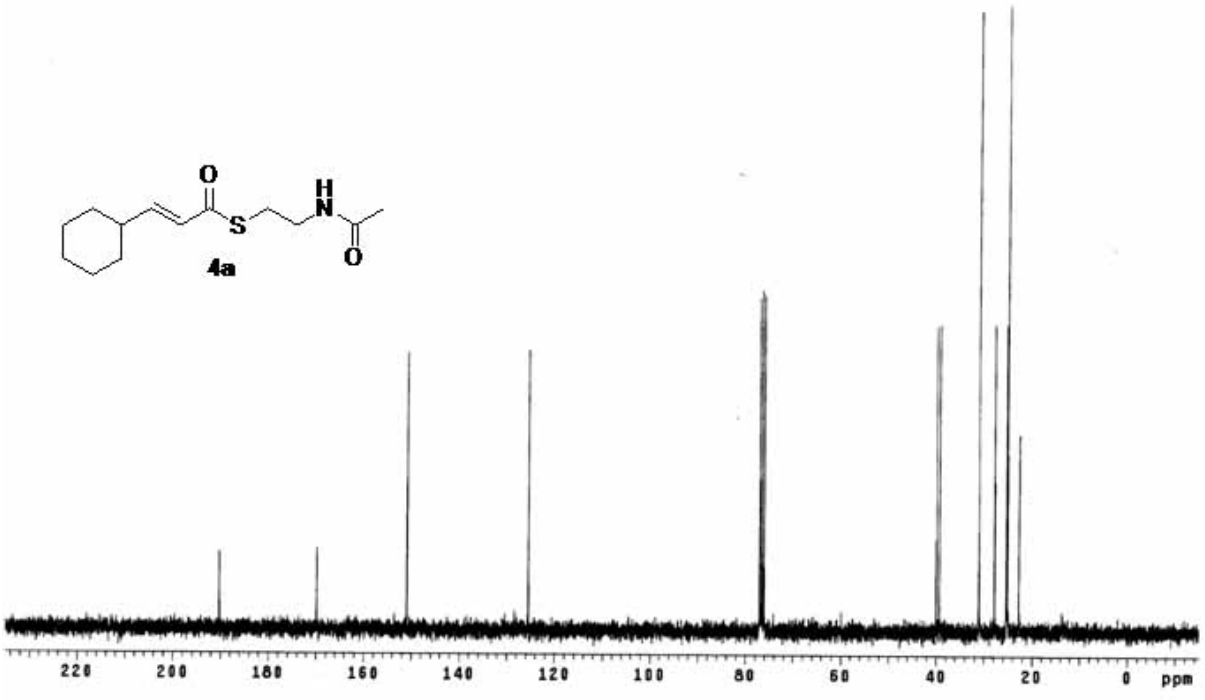
2.55
2.51
1.30
3.50
7.64
6.54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200
180
160
140
120
100
80
60
40
20
0
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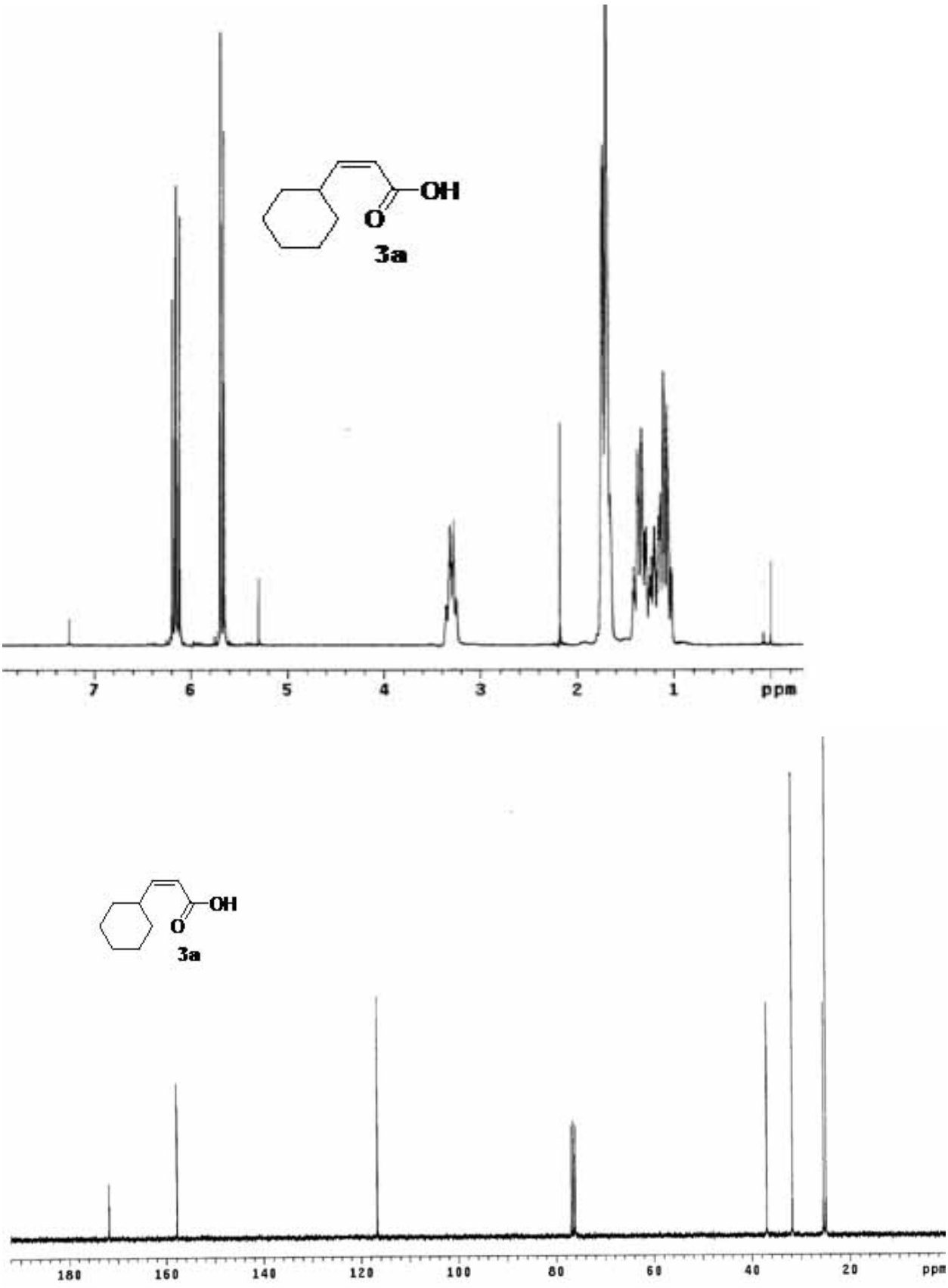
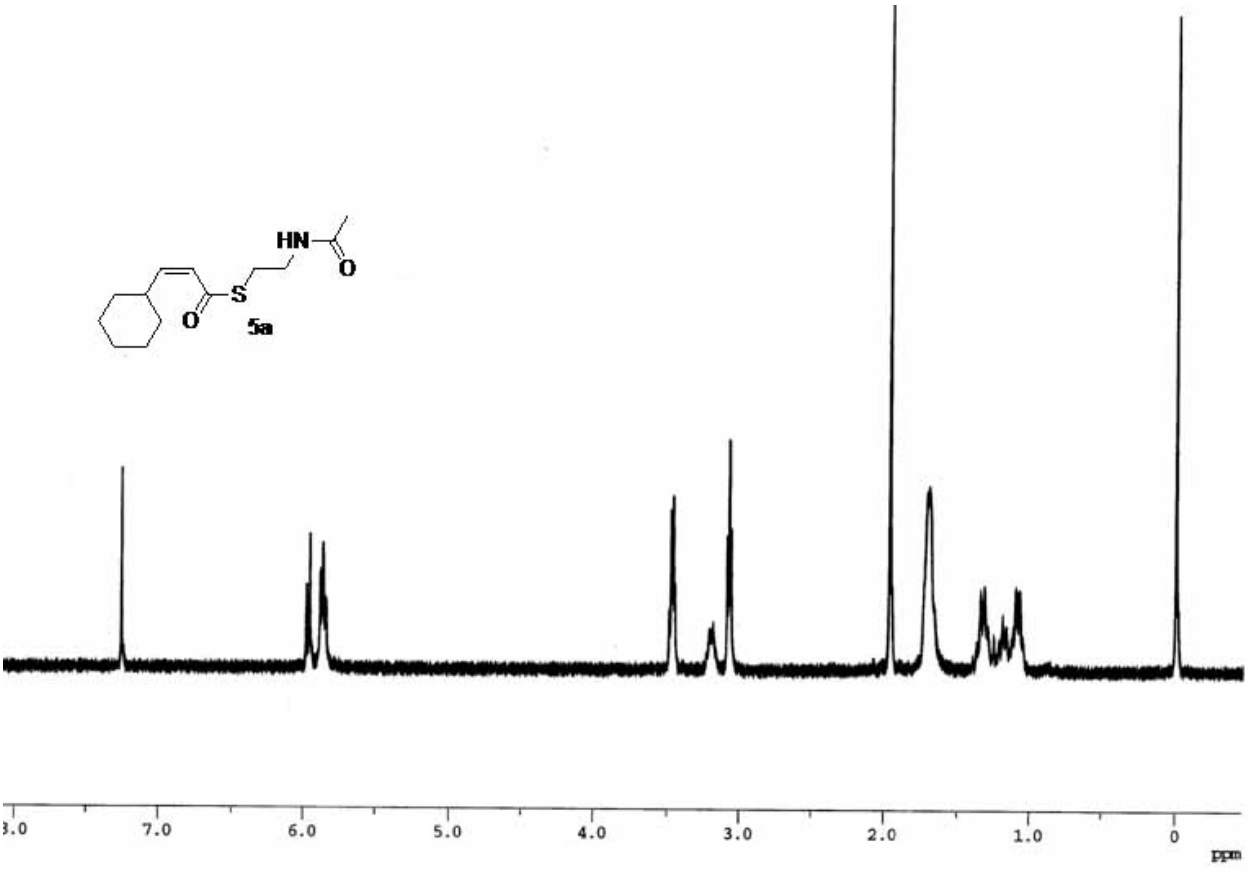
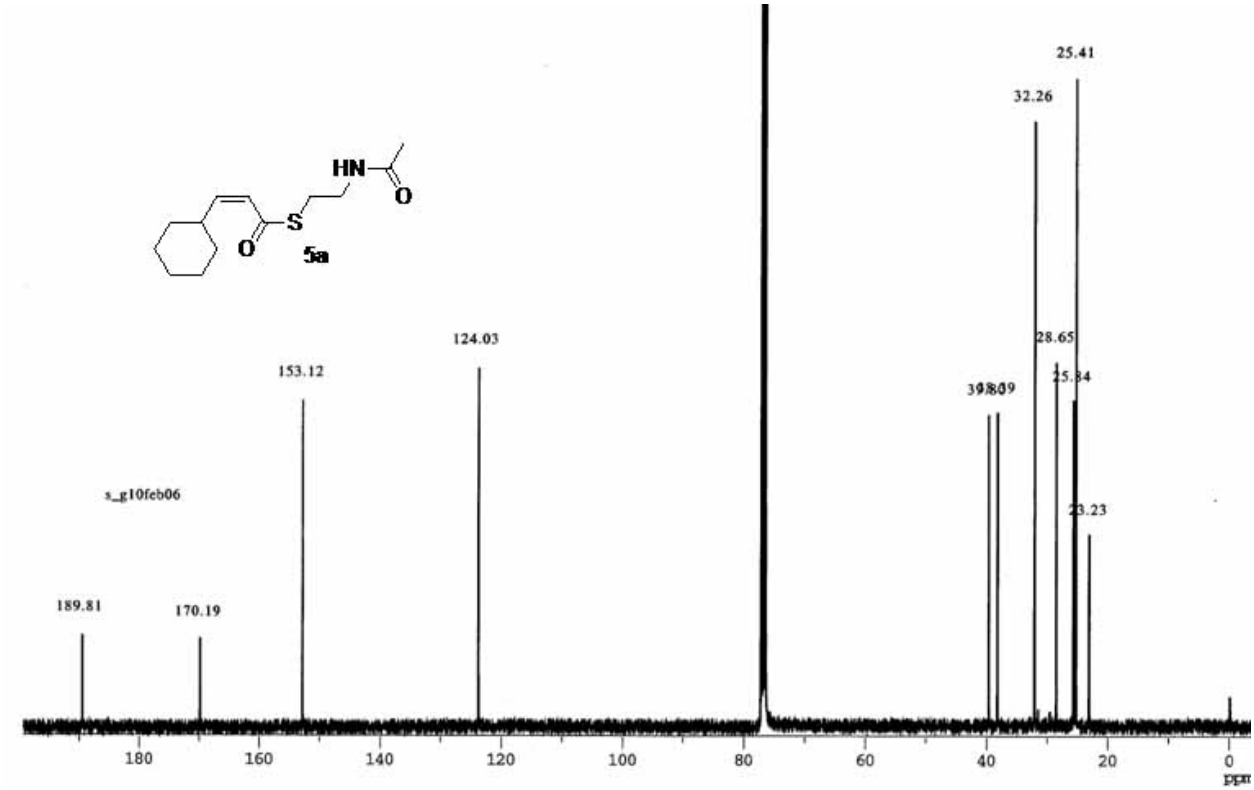

OH
O
3a
7
6
5
4
3
2
1
ppm
OH
O
3a
180
160
140
120
100
80
60
40
20
ppm


HN
O
S
O
5a
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
ppm


HN
O
S
O
5a
25.41
32.26
124.03
153.12
28.65
25.34
23.23
s_g10feb06
189.81
170.19
180
160
140
120
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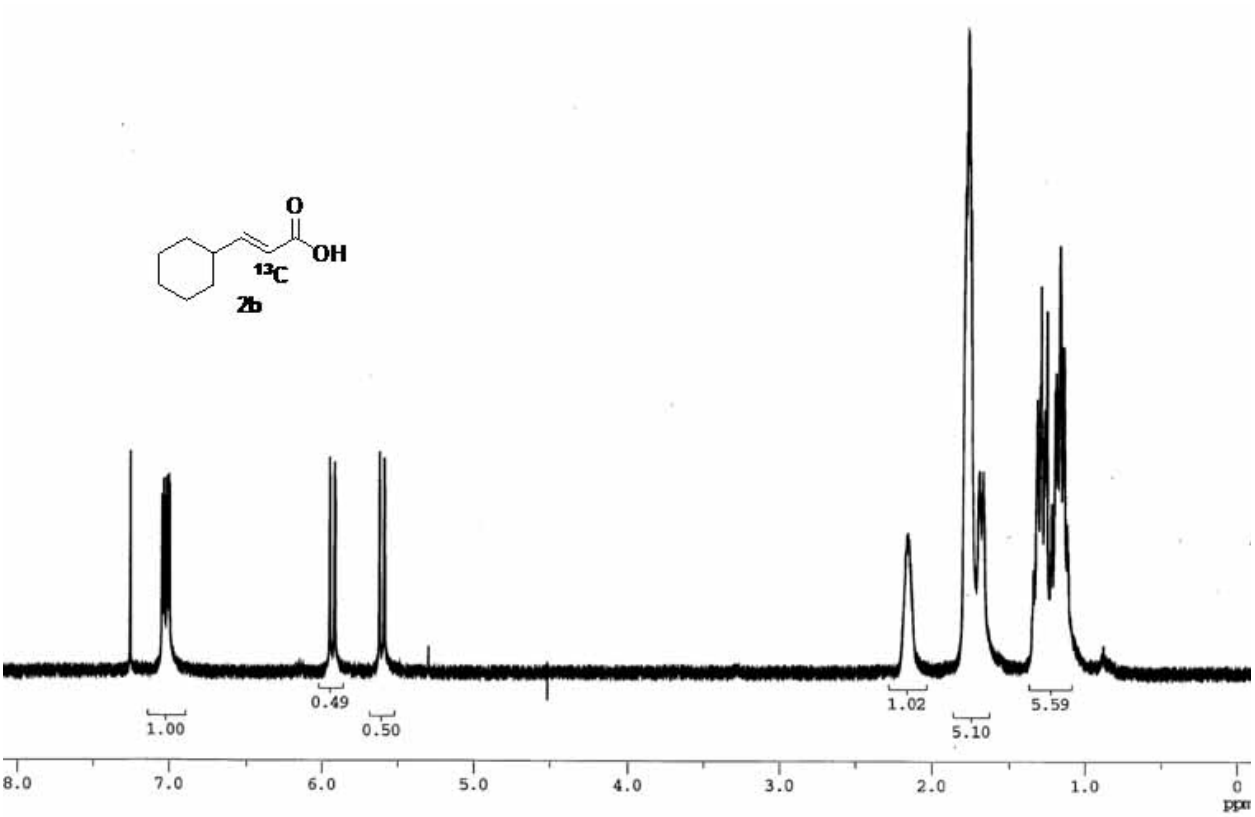
80
60
40
20
0
ppm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OH
13C
2b
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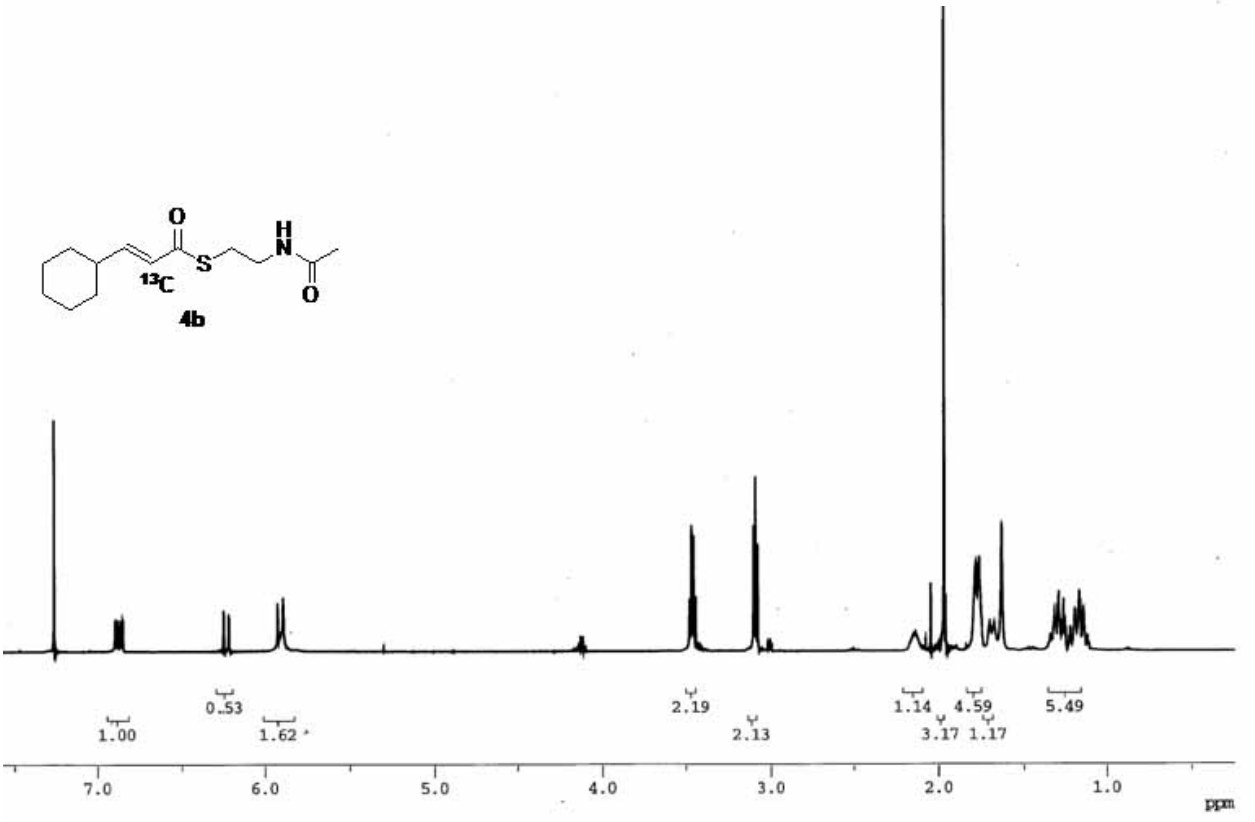
0.49
0.50
1.02
5.10
5.59
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
ppm


O
H
N
S
O
13C
4b
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0.53
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2.19
2.13
1.14
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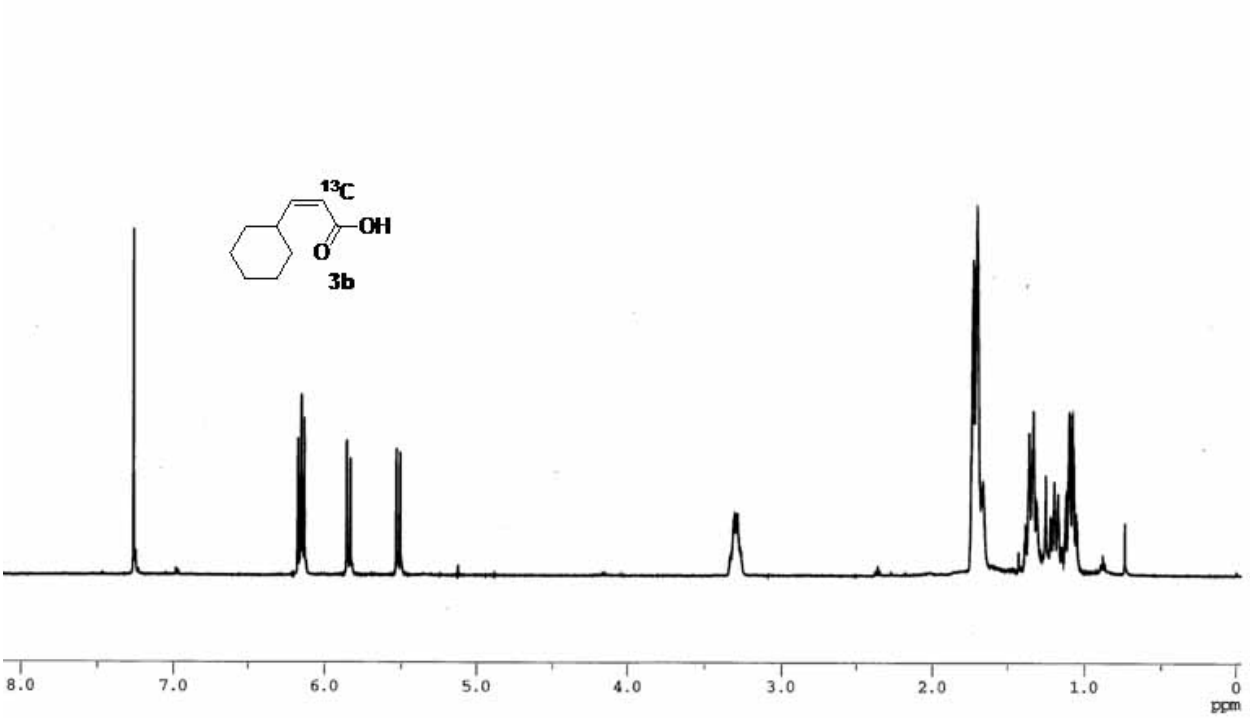
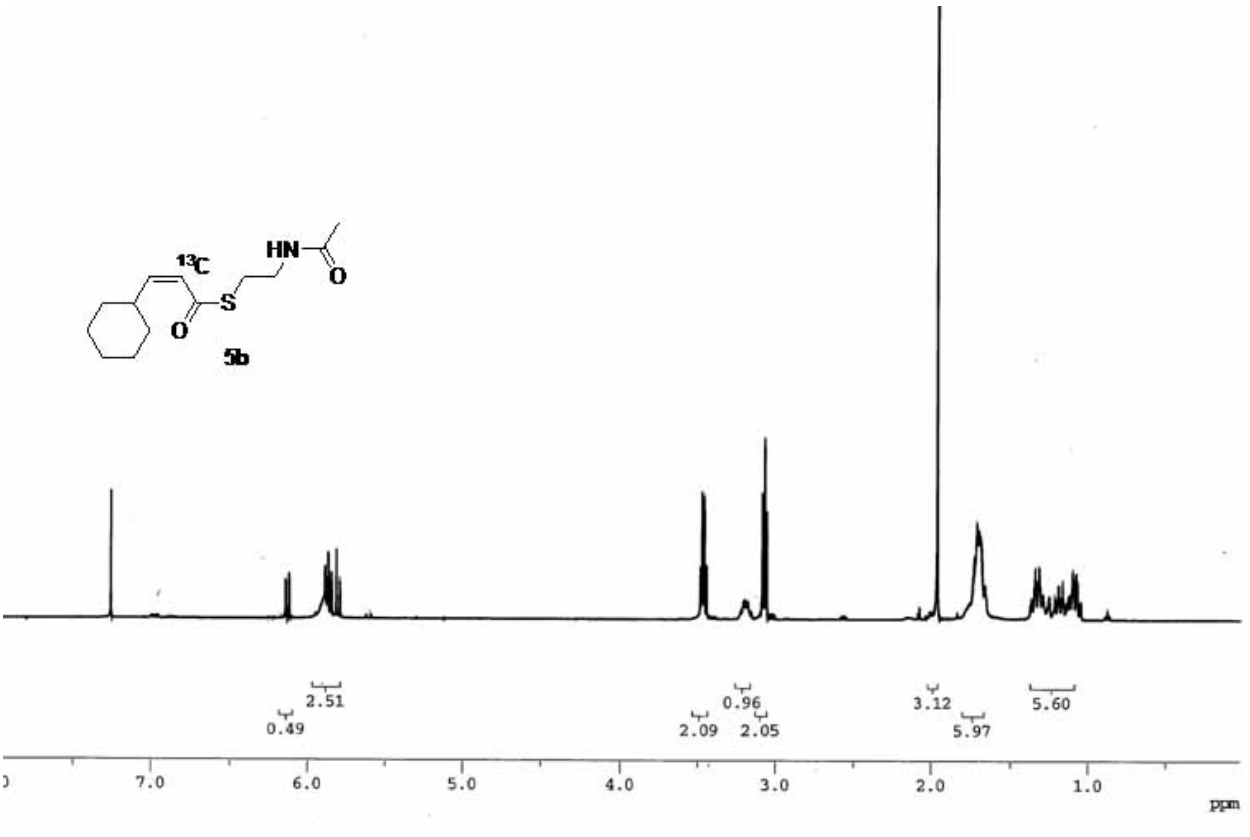
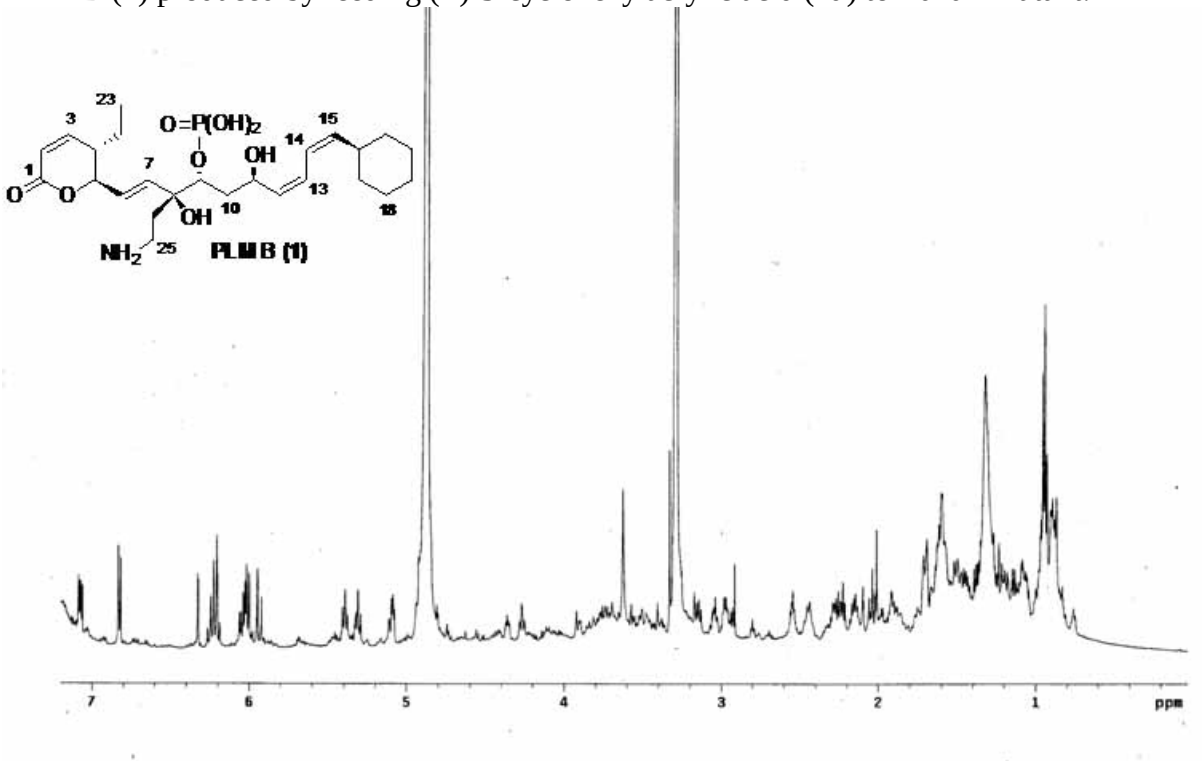
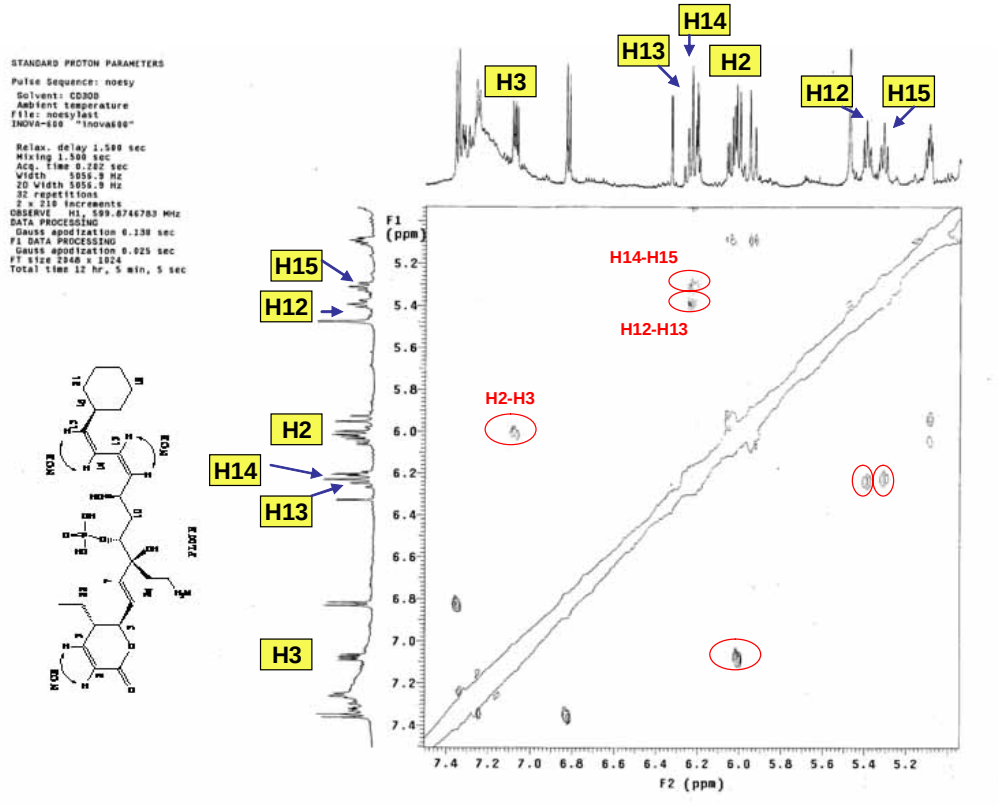
4.59
1.17
5.49
7.0
6.0
5.0
4.0
3.0
2.0
1.0
ppm


13C
OH
O
3b
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
ppm

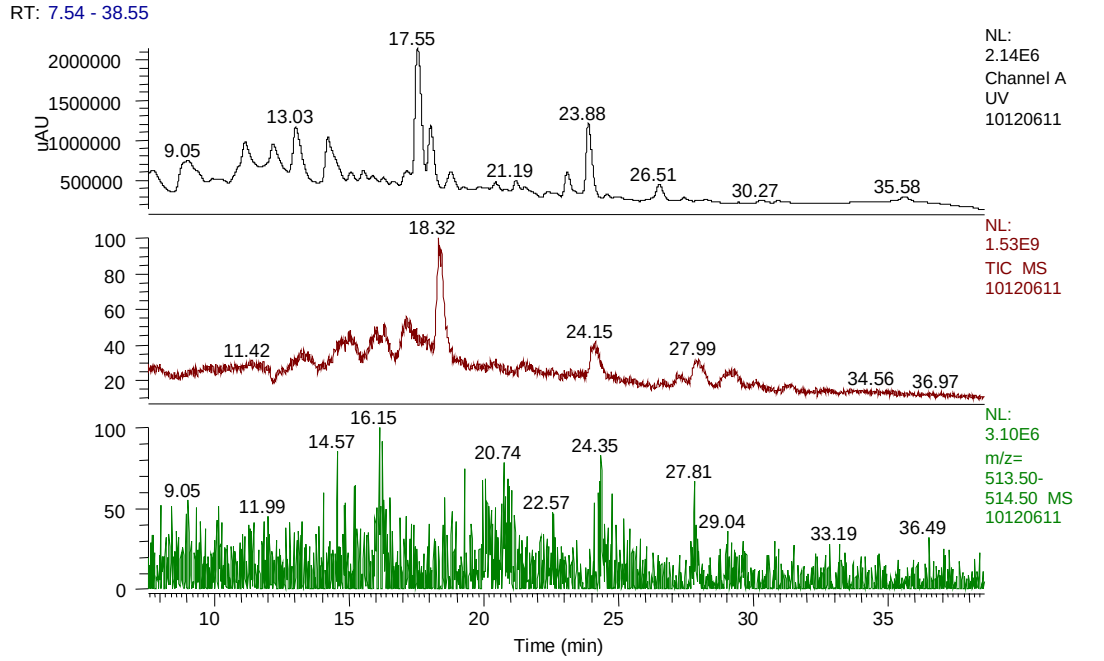

HN
O
13C
S
O
5b
0.49
2.51
2.09
0.96
2.05
3.12
5.97
5.60
7.0
6.0
5.0
4.0
3.0
2.0
1.0
ppm

PLM B (**1**) produced by feeding (*E*)-3-cyclohexylacrylic acid (**2a**) to Δ*chcA* mutant.

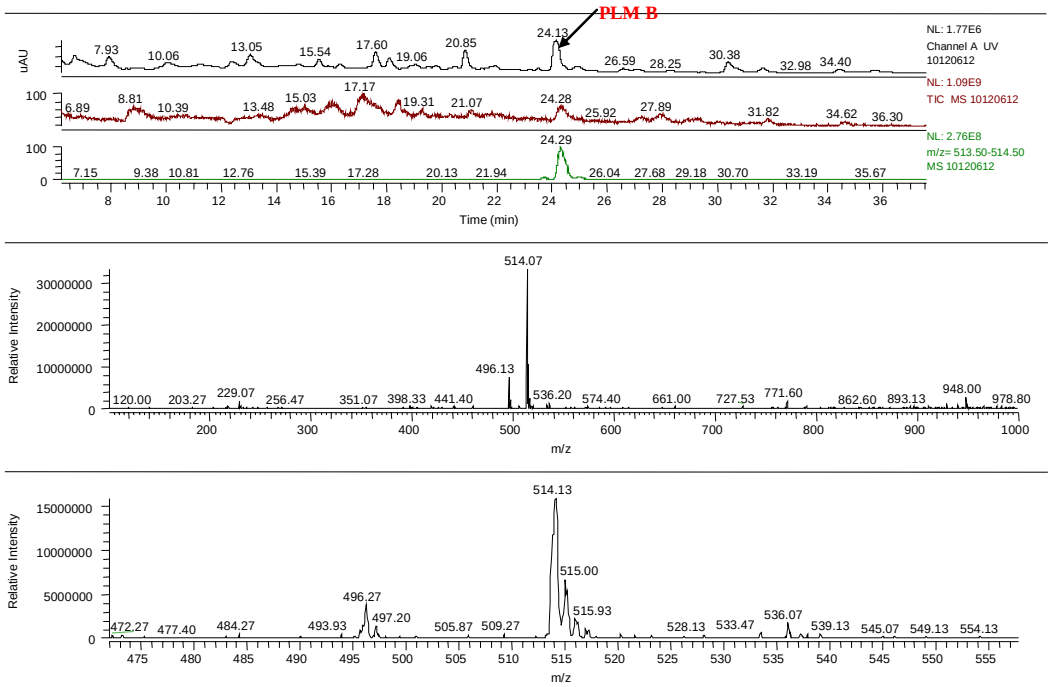




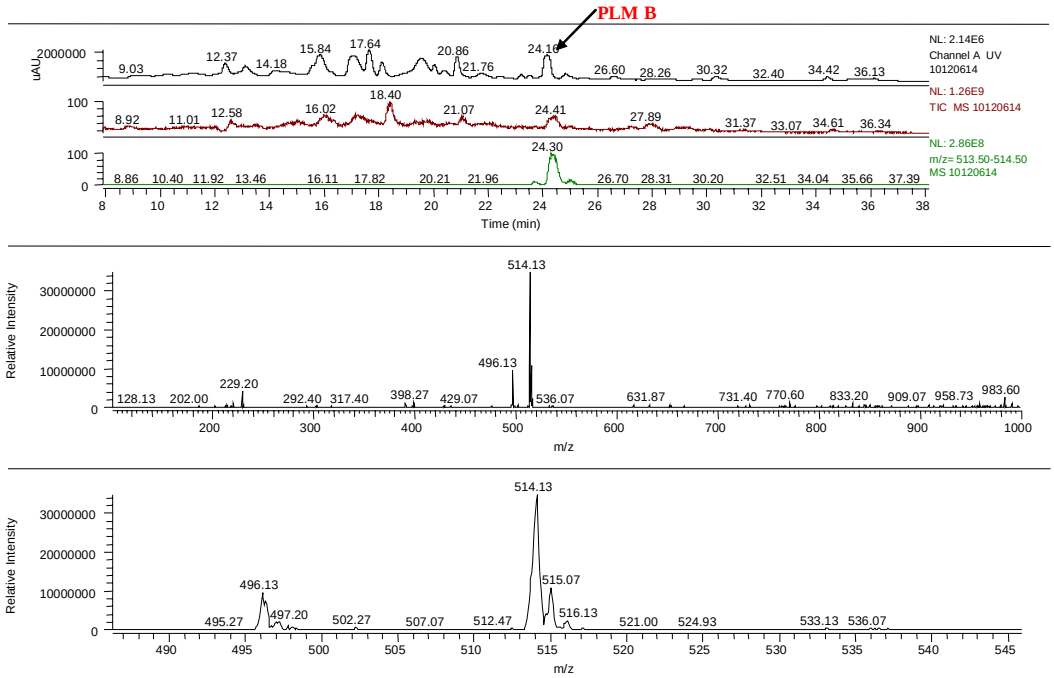
PLM B was not detected in NP3 mutant



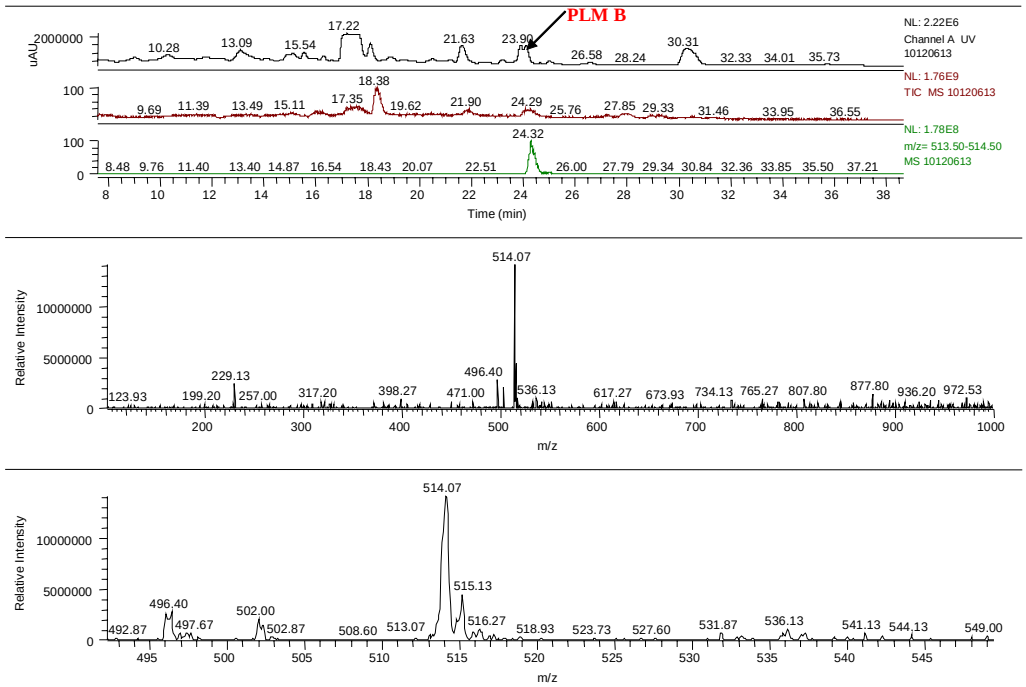
Production of PLM B in NP3 mutant by CHC supplementation



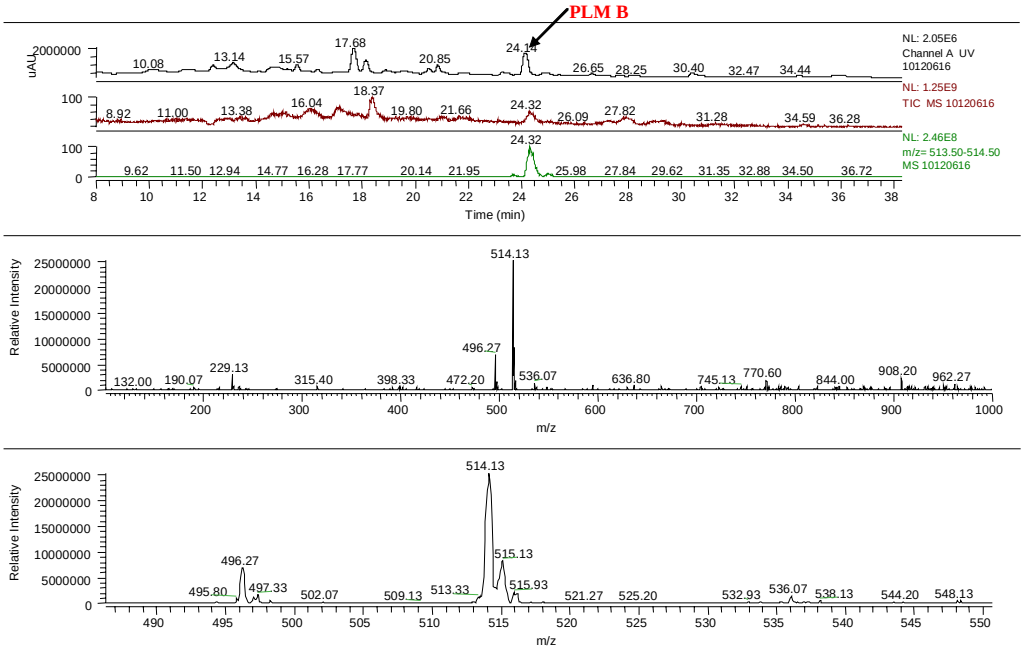
Production of PLM B in NP3 mutant by 2a supplementation



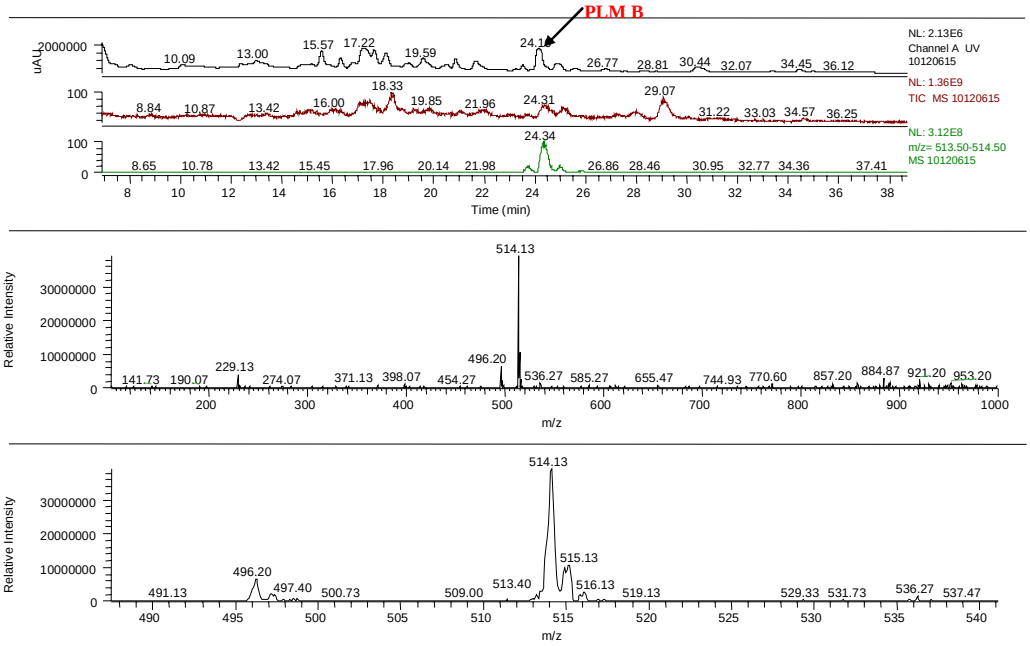
Production of PLM B in NP3 mutant by 3a supplementation



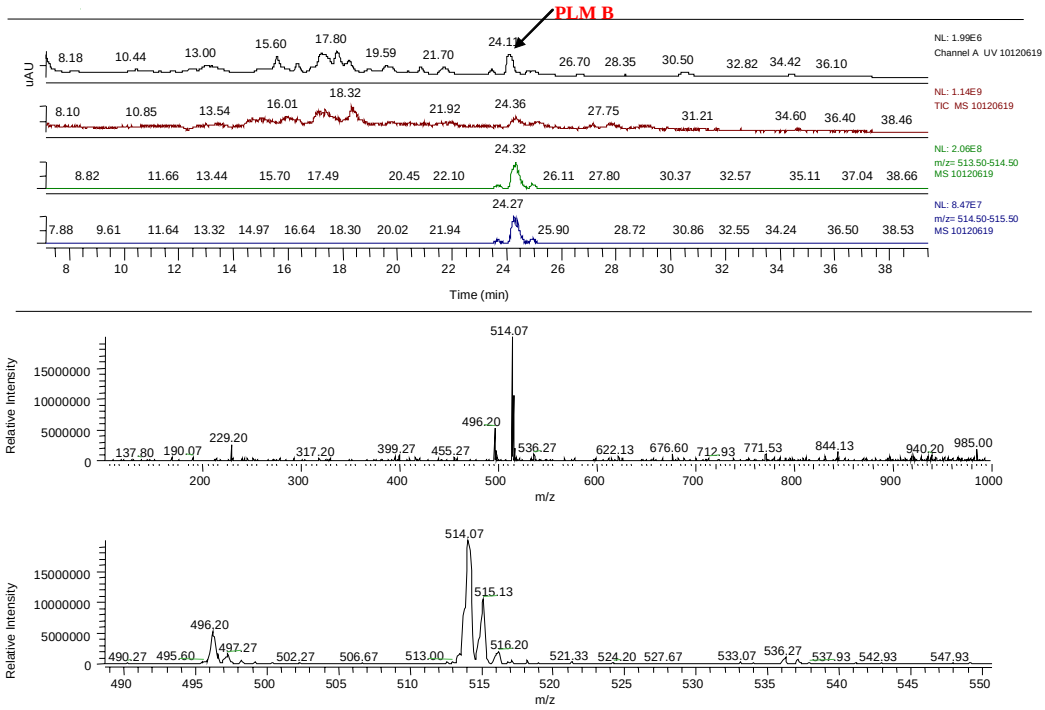
Production of PLM B in NP3 mutant by 4a supplementation



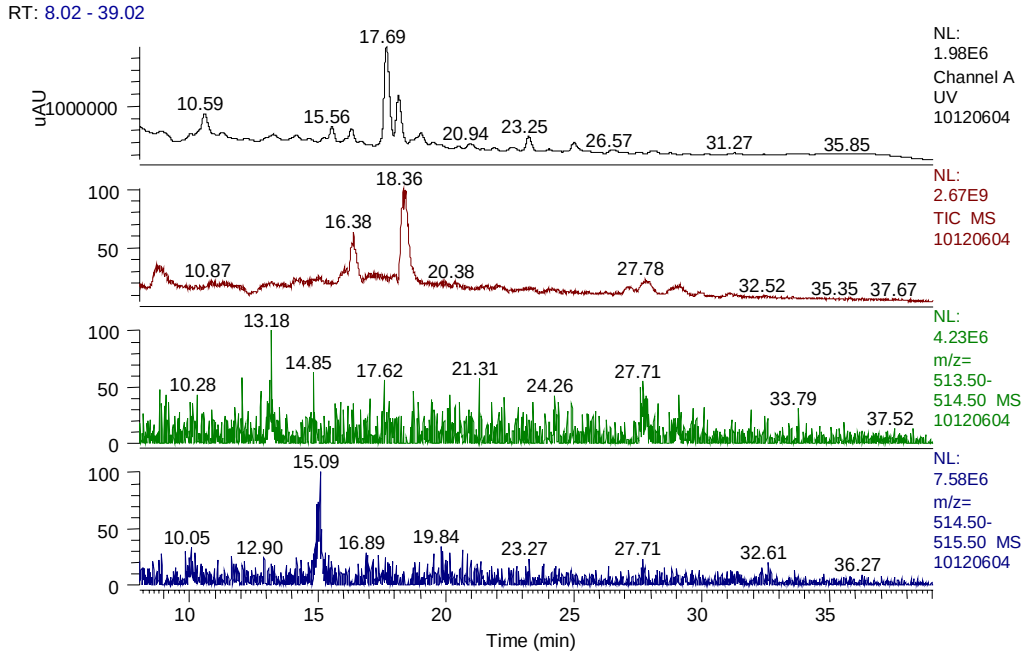
Production of PLM B in NP3 mutant by 5a supplementation



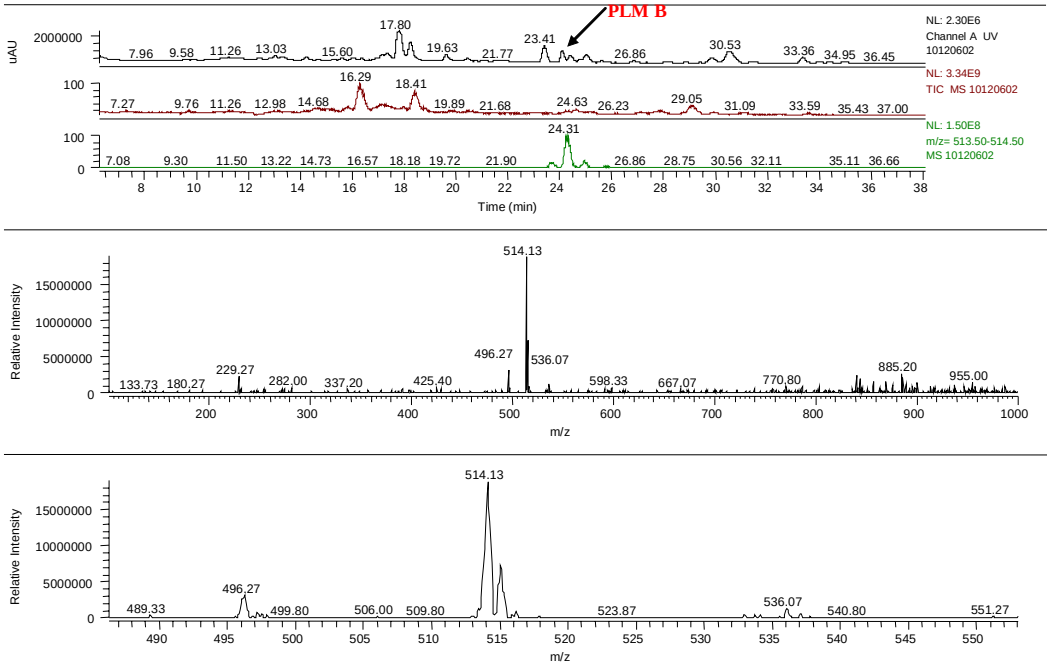
Production of PLM B in NP3 mutant by 5b supplementation



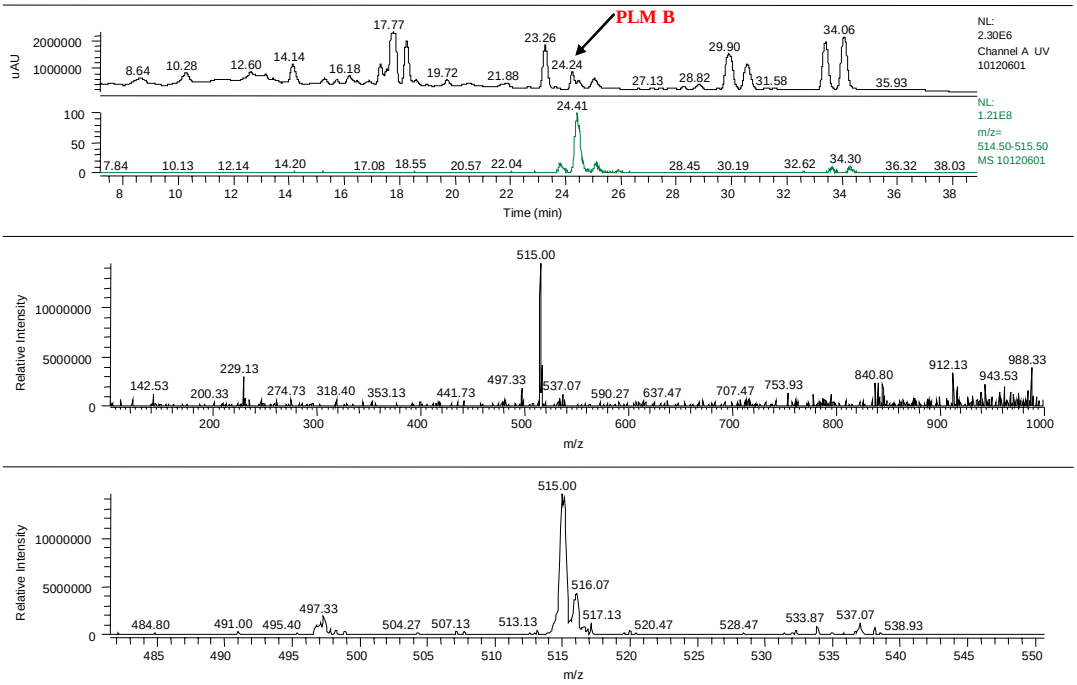
PLM B was not detected in NP9 mutant by CHC supplementation



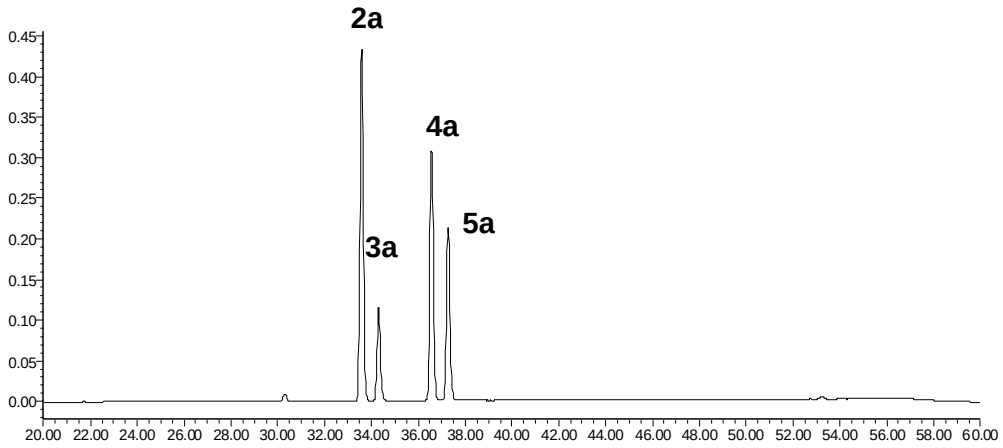
Production of PLM B in NP9 mutant by 5a supplementation



Production of PLM B in NP9 mutant by 5b supplementation

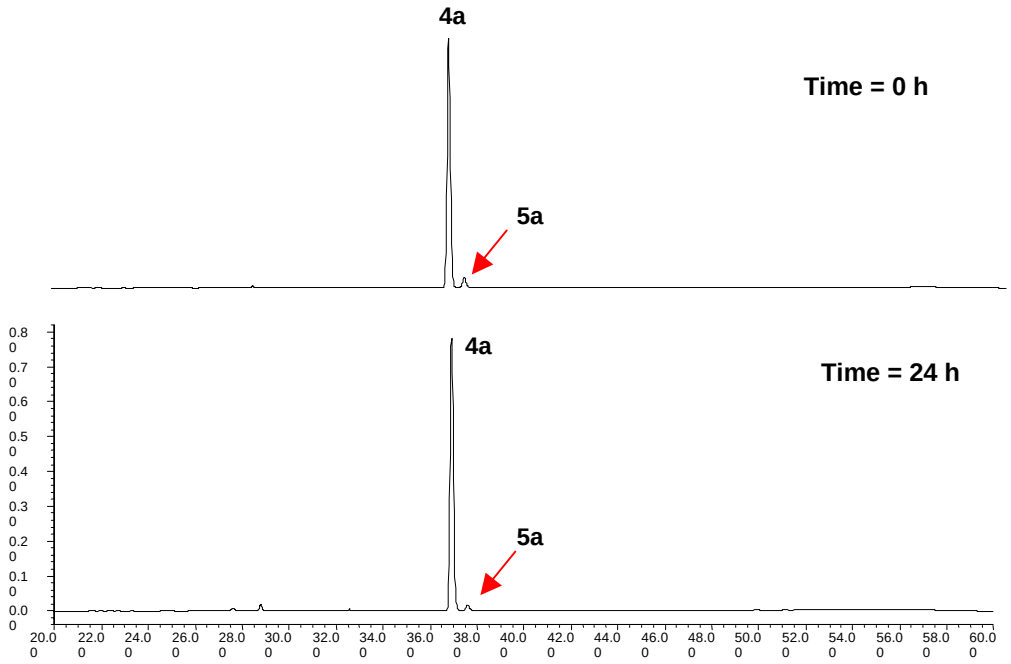


Standard (HPLC): mixture of 2a, 3a, 4a, and 5a



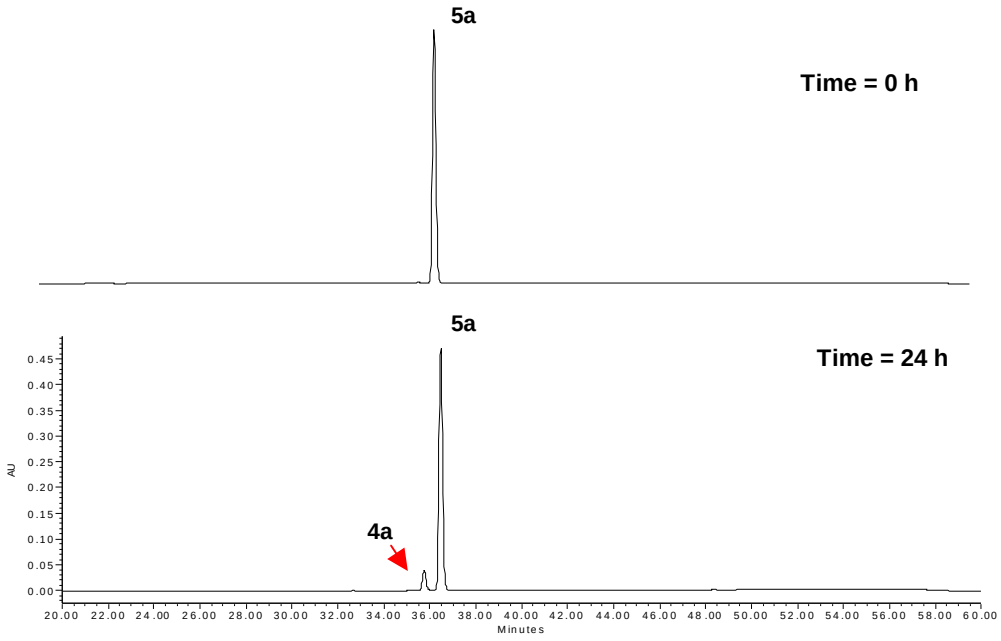
Stability of 4a at pH 7.0

There are trace levels of cis SNAC **5a** within the trans SNAC **4a** sample which could not be observed in the ^{1}H NMR but could be observed in both HPLC (~ 1%) and LC-MS as shown below. Incubation of **4a** + (1%) **5a** at pH = 7.0 (same pH as fermentation medium) for 24 hr at rt resulted in slight isomerization of the 1% **5a** to **4a**.



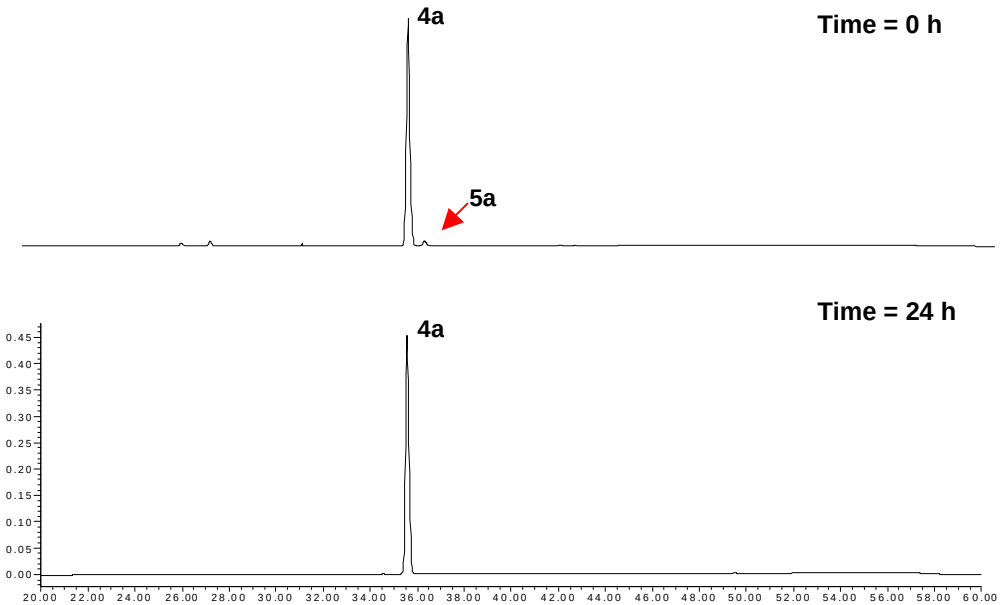
Stability of 5a at pH 7.0

Incubation of cis SNAC **5a** at pH = 7.0 for 24 hr at rt resulted in ~ 7% isomerization of **5a** to **4a**.



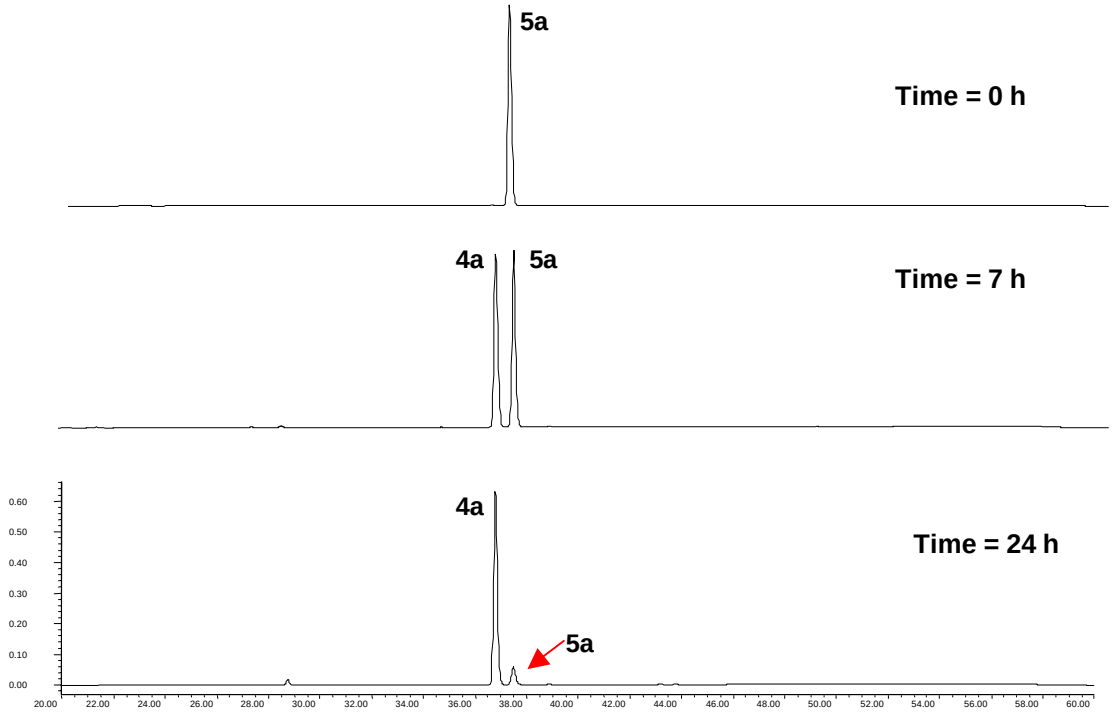
Stability of 4a at pH 8.0

Incubation of **4a** + (1%) **5a** at pH = 8.0 for 24 hr at rt resulted in complete isomerization of the 1% **5a** to **4a**.

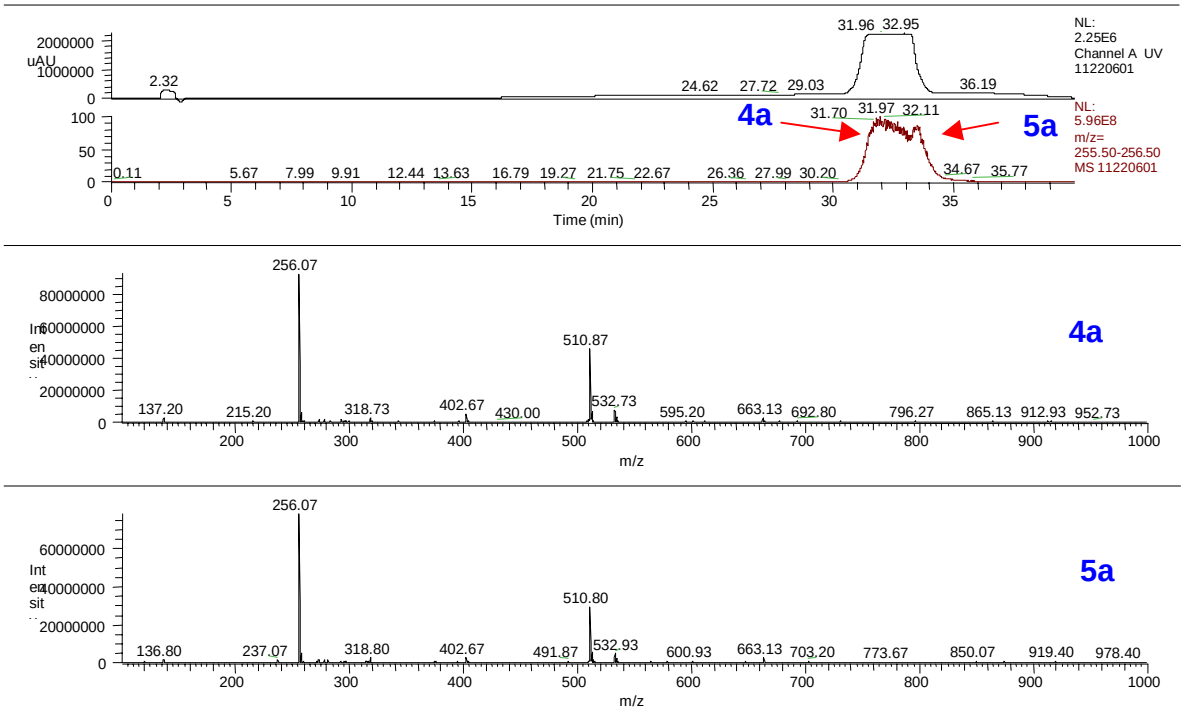


Stability of 5a at pH 8.0

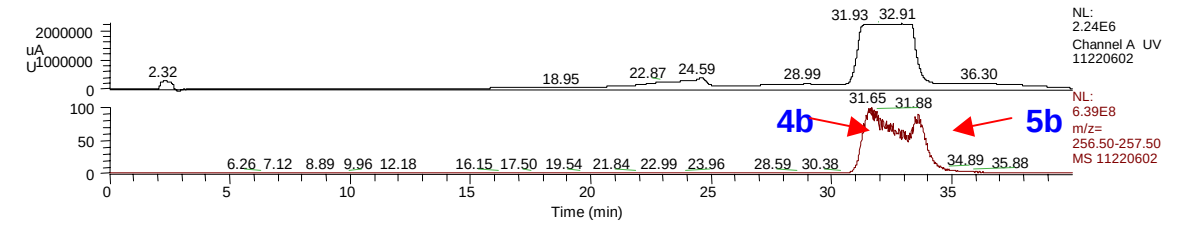
Incubation of cis SNAC **5a** at pH = 8.0 for 24 hr at rt resulted in ~ 87% isomerization of **5a** to **4a**.

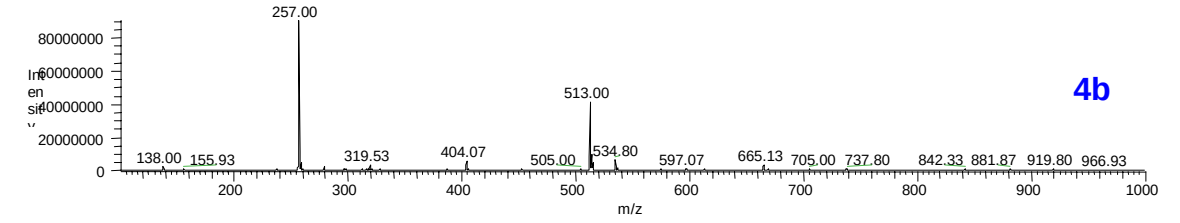


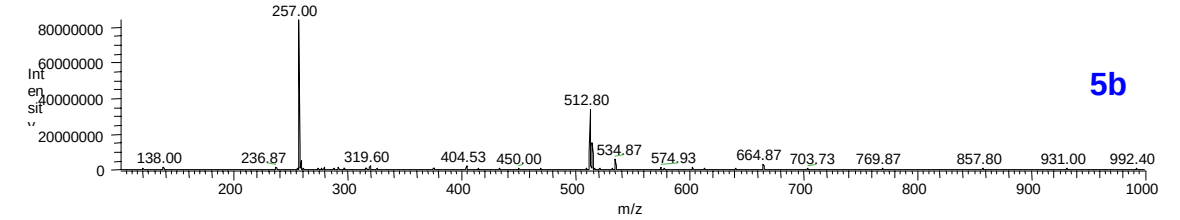
LC-MS of a standard synthetic sample of 4a + (~ 1%) 5a



LC-MS of a standard synthetic sample of 4b + (~1%) 5b







LC-MS of NP9 mutant supplemented with 4b (detection of remaining 4b &5b)