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for

In vitro Synthesis of Novel Enniatins: Probing the α-D-Hydroxy Carboxy Acid Binding Pocket of the Multienzyme Enniatin Synthetase ESyn

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I. Experimental

General techniques: All reactions were monitored by thin-layer chromatography carried out on 0.25 mm E.Merck silica gel plates (60F-254) using UV light as visualizing agent and Molybdat or Permanganate solution and heat as developing agents. E. Merck silica gel (60, particle size 0.040 – 0.063 mm) was used for flash column chromatography. Preparative thin-layer chromatography (PTLC) separations were carried out on 0.25, 0.5 or 1mm E. Merck silica gel plates (60F-254). NMR spectra were recorded on a Bruker Advance-400 instrument and calibrated using residual undeuterated solvent as an internal reference. The following abbreviations were used to explain the multiplicities: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; band, several overlapping signals; b, broad. Optical rotations were recorded on a Perkin-Elmer 241 polarimeter. High-resolution mass spectra (HRMS) were recorded on a Kratos MS 80 RFA mass spectrometer under electron impact ionization (EI) conditions.

Typical procedure for the enantioselective synthesis of *α*-D-hydroxy acids

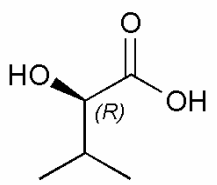
α-D-Amino acids; typical procedure: Boc-α-D-amino acid (1.0 mmol, 1.0 equiv) was dissolved in anhydrous CH_2Cl_2 (2 ml), and TFA (1 ml, 22.0 equiv) was added. After stirring for 3 hours at room temperature, the reaction has completed, followed by TLC. When complete, the reaction mixture was treated with saturated aqueous Na_2CO_3 solution until pH 9 was achieved. Then, it was extracted with CH_2Cl_2 (2 x 30 mL), and the combined organic phase where dried over $MgSO_4$ followed by evaporation of the solvent, obtaining the unprotected amino acid **8** (90-98%) as a white solid.

*α-D-Hydroxy carboxylic acids; typical procedure***:** α-L-amino acid (10.0 mmol, 1.0 equiv) was dissolved in 0.5 M H_2SO_4 (40 ml, 20.00 mmol, 2.0 eqiv). This solution was heated to 60 °C and a solution of sodium nitrite (4.14 g, 60.0 mmol, 6.0 equiv) in H_2O (13.5 ml) was added slowly while stirring, the temperature was maintained. The reaction was stirred at 60 °C for further 3 h, and then allowed to cool down to room temperature and stirred at this temperature for 24 h (followed by TLC to completion). The reaction mixture was extracted with diethyl ether (3 x 50 mL), the organic phases were combined, washed with brine (50 ml) and dried over anhydrous sodium sulfate. The drying agent was filtered off, and the ether was evaporated in vacuo. The residu-

al mass was recrystallized from hexane-ether to give, respective α-L-hydroxy carboxylic acid.

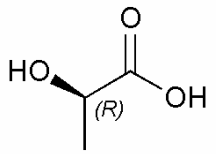
Analytical and spectral data of *α*-D-hydroxy acids

*(*R*)-2-hydroxy-3-methyl-butyric acid (**1**)*



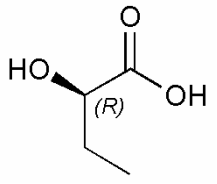
Yield: 90%; pale white solid; ^{1}H NMR (400 MHz, DMSO): δ 0.81 (d, ^{1}J = 6.85, 3H, CH_3), 0.88 (d, ^{1}J = 6.85, 3H, CH_3), 1.87-1.93 (m, 1H, CH), 3.72 (d, ^{1}J = 4.57, 1H, CH); ^{13}C NMR (100 MHz, DMSO): δ 175.3, 74.5, 31.4, 18.9, 16.7; IR (film): 3423, 3171, 2967, 2936, 2878, 2639, 1720, 1468, 1371, 1254, 1215, 1178, 1137, 1066, 1028; MS: m/z (%) = 119 (5), 99 (2), 85 (8), 76 (100), 55 (40); HRMS m/z calcd for $C_5H_{11}O_3$: [MH+] 119.0708; found: 119.0710.

*(*R*)-2-hydroxy-propionic acid (**2**)*



Yield: 87%; yellow oil; ^{1}H NMR (400 MHz, DMSO): δ 1.21 (d, ^{1}J = 6.85, 3H, CH_3), 2.95 (q, ^{1}J = 6.90, 1H, CH); ^{13}C NMR (100 MHz, DMSO): δ 176.3, 65.7, 20.4; IR (film): 3206, 2990, 2943, 2632, 1729, 1646, 1455, 1376, 1277, 1203, 1124, 1043; MS: m/z (%) = 90 (5), 74 (15), 56 (100); HRMS m/z calcd for $C_3H_6O_3$: 90.0317; found: 90.0329.

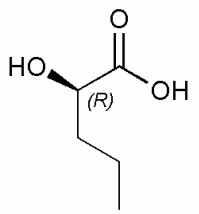
*(*R*)-2-hydroxy-butyric acid (**3**)*



Yield: 79%; yellowish solid; ^{1}H NMR (400 MHz, DMSO): δ 0.85 (t, ^{1}J = 7.46, 3H, CH_3), 1.48-1.65 (m, 2H, CH_2), 3.85-3.88 (m, 1H, CH); ^{13}C NMR (100 MHz, DMSO): δ 175.7, 70.7, 27.0, 9.5; IR (film): 3449, 2971, 2939, 2883, 2635, 1718, 1460, 1381,

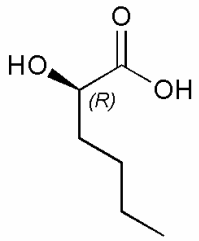
1360, 1333, 1238, 1214, 1128, 1059; MS: m/z (%) = 105 (80), 87 (5), 77 (20), 59 (100); HRMS m/z calcd for $C_4H_9O_3$: [MH+] 105.0551; found: 105.0559.

*(*R*)-2-hydroxy-pentanoic acid (**4**)*



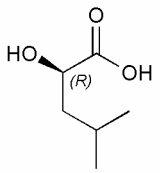
Yield: 95%; pale white solid; ^{1}H NMR (400 MHz, DMSO): δ 0.85 (t, ^1J = 7.32, 3H, CH_3), 1.29-1.38 (m, 2H, CH_2), 1.44-1.61 (m, 2H, CH_2), 3.91 (dd, ^1J = 7.79, ^2J = 4.57, 1H, CH); ^{13}C NMR (100 MHz, DMSO): δ 175.9, 69.3, 36.0, 17.9, 13.7; IR (film): 3319, 2963, 2936, 2876, 2645, 1722, 1651, 1467, 1381, 1467, 1381, 1216, 1132, 1075; MS: m/z (%) = 119 (5), 100 (10), 73 (80), 55 (100); HRMS m/z calcd for $C_5H_{11}O_3$: [MH+] 119.0708; found: 119.0712.

*(*R*)-2-hydroxy-hexanoic acid (**5**)*



Yield: 91%; white solid. ^{1}H NMR (400 MHz, DMSO): δ 0.84 (t, ^1J = 7.19, 3H, CH_3), 1.21-1.32 (m, 4H, $(CH_2)_2$), 1.46-1.64 (m, 2H, CH_2), 3.89 (dd, ^1J = 7.79, ^2J = 4.57, 1H, CH); ^{13}C NMR (100 MHz, DMSO): δ 175.9, 69.5, 33.6, 26.9, 21.9, 13.9; IR (film): 3459, 2957, 2930, 2874, 2862, 2654, 2589, 1731, 1648, 1467, 1460, 1380, 1271, 1242, 1208, 1134, 1087, 1066, 1056; MS: m/z (%) = 132 (5), 103 (3), 87 (48), 76 (17), 69 (100), 57 (15); HRMS m/z calcd for $C_6H_{12}O_3$: 132.0786; found: 132.00782.

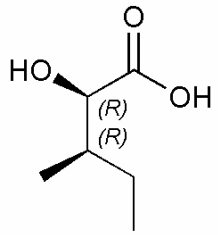
*(*R*)-2-hydroxy-4-methyl-pentanoic acid (**7**)*



Yield: 88%; yellow solid; ^{1}H NMR (400 MHz, DMSO): δ 0.86 (d, ^1J = 6.31, 6H, $(CH_3)_2$), 1.35-1.47 (m, 2H, CH_2), 1.68-1.79 (m, 1H, CH), 3.92 (dd, ^1J = 8.73, ^2J =

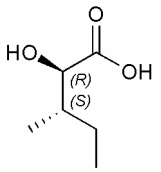
4.48, 1H, CH); ^{13}C NMR (100 MHz, DMSO): δ 176.4, 68.2, 42.9, 23.9, 23.2, 21.5; IR (film): 3186, 2959, 2938, 2872, 2630, 1723, 1651, 1470, 1456, 1388, 1369, 1266, 1271, 1141, 1086; MS: m/z (%) = 132 (5), 113 (8), 99 (15), 87 (75), 76 (35), 69 (100), 57 (22); HRMS m/z calcd for $C_6H_{12}O_3$: 132.0786; found: 132.0788.

*(*R*)-2-hydroxy-3-methyl-pentanonic acid (**8**)*



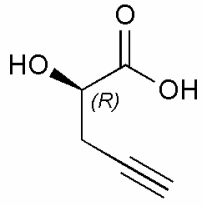
Yield: 91%; white solid. ^{1}H NMR (400 MHz, DMSO): δ 0.82 (t, ^1J = 7.80, 3H, CH_3), 0.85 (d, ^1J = 6.98, 3H, CH_3), 1.10-1.17 (m, 1H, CH_2), 1.36-1.42 (m, 1H, CH_2), 1.62-1.69 (m, 1H, CH), 3.76 (d, ^1J = 4.97, 1H, CH); ^{13}C NMR (100 MHz, DMSO): δ 175.4, 74.0, 38.1, 23.7, 15.4, 11.5; IR (film): 3314, 2966, 2937, 2879, 2625, 1718, 1649, 1463, 1381, 1273, 1242, 1216, 1135, 1071, 1045, 1018; MS: m/z (%) = 133 (6), 115 (4), 87 (30), 76 (100), 69 (15), 57 (35); HRMS m/z calcd for $C_6H_{13}O_3$: [MH+] 133.0864; found: 133.0861.

*(*allo*)-2-hydroxy-3-methyl-pentanonic acid (**9**)*



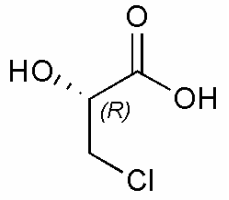
Yield: 82%; white solid; ^{1}H NMR (400 MHz, DMSO): δ 0.76 (d, ^1J = 6.85, 3H, CH_3), 0.85 (t, ^1J = 7.46, 3H, CH_3), 1.11-1.22 (m, 1H, CH_2), 1.34-1.45 (m, 1H, CH_2), 1.64-1.70 (m, 1H, CH), 3.89 (d, ^1J = 3.63, 1H, CH); ^{13}C NMR (100 MHz, DMSO): δ 175.8, 72.1, 37.9, 25.6, 13.8, 11.7; IR (film): 3314, 2966, 2937, 2879, 2625, 1718, 1649, 1463, 1381, 1273, 1242, 1216, 1135, 1071, 1045, 1018; MS: m/z (%) = 133 (22), 115 (10), 105 (32), 97 (12), 87 (100), 76 (44), 69 (54), 57 (38); HRMS m/z calcd for $C_6H_{13}O_3$: [MH+] 133.0864; found: 133.0863.

*(*R*)-2-hydroxy-pent-4-ynoic acid (**12**)*



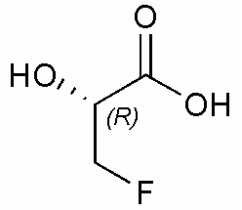
Yield: 78%; pale brown solid; ^{1}H NMR (400 MHz, DMSO): δ 2.40-2.53 (m, 2H, CH_2), 2.78 (m, 1H, CH), 4.05 (dd, ^{1}J = 6.45, ^{2}J = 5.37, 1H, CH); ^{13}C NMR (100 MHz, DMSO): δ 173.9, 80.8, 72.7, 68.7, 24.2; IR (film): 3290, 2926, 2633, 2122, 1726, 1422, 1361, 1329, 1205, 1093, 1027, 960; MS: m/z (%) = 115 (100), 109 (5), 97 (38), 91 (9), 87 (21), 69 (25), 53 (7); HRMS m/z calcd for $C_5H_7O_3$: [MH+] 115.0395; found: 115.0402.

*(*R*)-3-chloro-2-hydroxy-propionic acid (**14**)*



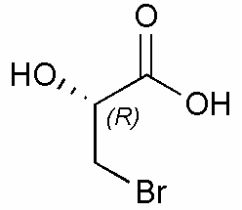
Yield: 75%; yellowish solid; ^{1}H NMR (400 MHz, DMSO): δ 3.96 (m, 2H, CH_2), 4.88 (dd, ^{1}J = 5.3, ^{2}J = 11.3, 1H, CH); ^{13}C NMR (100 MHz, DMSO): δ 172.5, 70.1, 47.2; IR (film): 3181, 2976, 2930, 2607, 1731, 1430, 1369, 1276, 1224, 1184, 1099, 910, 857, 772, 678; MS: m/z (%) = 107 (50), 97 (10), 89 (15), 71 (13), 62 (100), 55 (7); HRMS m/z calcd for $C_3H_4O_2Cl$: [M-OH] 107.9899; found: 107.9900.

*(*R*)-3-fluoro-2-hydroxy-propionic acid (**15**)*



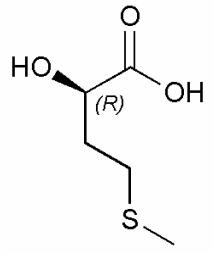
Yield: 65%; yellowish solid; ^{1}H NMR (400 MHz, DMSO): δ 4.19 (dd, ^{1}J = 4.30, ^{2}J = 2.96, 1H, CH), 4.27 (dd, ^{1}J = 4.30, ^{2}J = 2.96, 1H, CH), 4.48 (dd, ^{1}J = 5.44, ^{2}J = 3.69, 1H, CH_2), 4.59 (dd, ^{1}J = 4.90, ^{2}J = 3.69, 1H, CH_2); ^{13}C NMR (100 MHz, DMSO): δ 172.4, 85.9, 84.3, 69.8, 69.6; IR (film): 3378, 3196, 2966, 2925, 2855, 2619, 2564, 1731, 1630, 1561, 1459, 1379, 1237, 1208, 1122, 1063, 999; MS: m/z (%) = 109 (5), 97 (10), 81 (15), 73 (20), 69 (25), 63 (100), 55 (19), 45 (40), 40 (85); HRMS m/z calcd for $C_3H_6FO_3$: [MH+] 109.0301; found: 109.0304.

*(*R*)-3-bromo-2-hydroxy-propionic acid (**16**)*



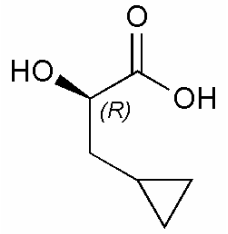
Yield: 79%; brownish solid; ^{1}H NMR (400 MHz, DMSO): δ 3.52 (dd, ^{1}J = 10.68, ^{2}J = 4.37, 1H, CH_2), 3.56 (dd, ^{1}J = 10.61, ^{2}J = 4.43, 1H, CH_2), 4.31 (t, ^{1}J = 4.50, 1H, CH); ^{13}C NMR (100 MHz, DMSO): δ 172.5, 69.7, 36.2; IR (film): 3251, 3041, 2975, 2927, 2856, 2623, 1737, 1417, 1338, 1275, 1172, 1104, 976; MS: m/z (%) = 150 (25), 125 (100), 107 (8), 101 (8), 71 (35), 55 (5); HRMS m/z calcd for $C_3H_3O_2Br$: [M-H_2O] 149.9316; found: 149.9312.

*(*R*)-2-hydroxy-4-methylsulfanyl-butyric acid (**17**)*



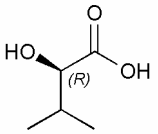
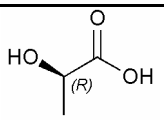
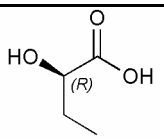
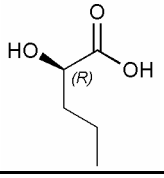
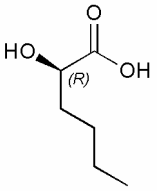
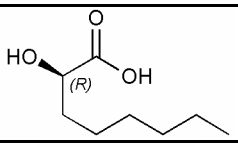
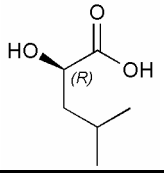
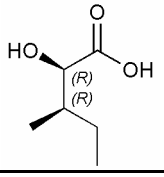
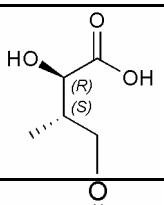
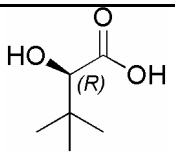
Yield: 89%; yellow oil; ^{1}H NMR (400 MHz, DMSO): δ 1.69-1.78 (m, 1H, CH_2), 1.82-1.91 (m, 1H, CH_2), 2.02 (s, 3H, CH_3), 2.62 (t, ^{1}J = 6.65, 2H, CH_2), 4.02 (dd, ^{1}J = 8.53, ^{2}J = 3.96, 1H, CH); ^{13}C NMR (100 MHz, DMSO): δ 175.5, 68.4, 33.6, 29.3, 14.6; IR (film): 3324, 2969, 2919, 2848, 2635, 1722, 1429, 1366, 1265, 1221, 1171, 1092; MS: m/z (%) = 150 (75), 132 (10), 120 (20), 105 (15), 89 (11), 75 (50), 61 (100), 57 (20); HRMS m/z calcd for $C_5H_{10}O_3S$: 150.0350; found: 150.0350.

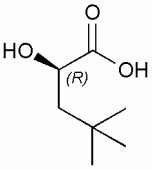
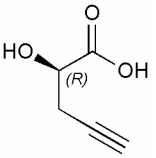
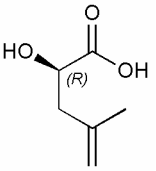
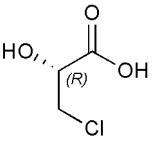
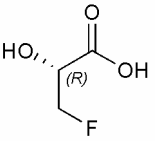
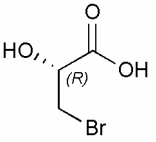
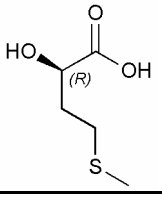
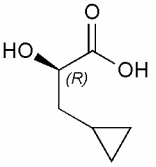
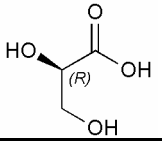
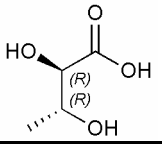
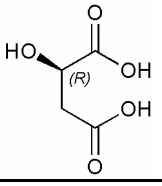
*(*R*)-3-cyclopropyl-2-hydroxy-propionic acid (**18**)*

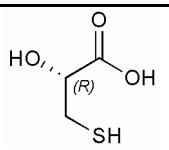
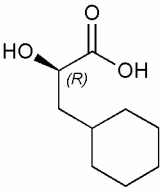
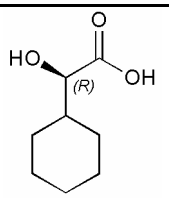
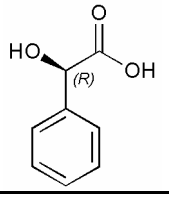
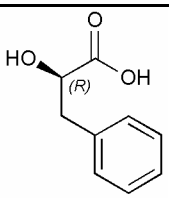
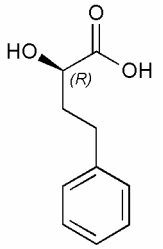
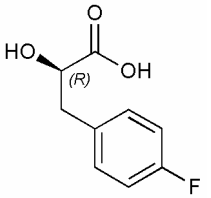
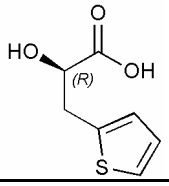
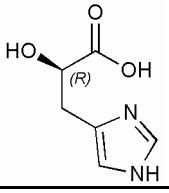


Yield: 81%; white solid; ^{1}H NMR (400 MHz, DMSO): δ 0.02-0.10 (m, 2H, CH_2), 0.32-0.45 (m, 2H, CH_2), 0.76-0.86 (m, 1H, CH), 1.37-1.44 (m, 1H, CH_2), 1.48-1.55 (m, 1H, CH_2), 3.95 (dd, ^{1}J = 7.46, ^{2}J = 4.77, 1H, CH); ^{13}C NMR (100 MHz, DMSO): δ 175.8, 70.2, 38.9, 7.3, 4.5, 3.9; IR (film): 3324, 3081, 3004, 2942, 2920, 2637, 2063, 1721, 1649, 1429, 1365, 1233, 1217, 1148, 1085, 1052; MS: m/z (%) = 130 (8), 112 (5), 97 (15), 84 (20), 76 (55), 67 (23), 55 (100); HRMS m/z calcd for $C_6H_{10}O_3$: 130.0629; found: 130.0621.

Tabel S1. α-D-Hydroxy acids used for in vitro assay.

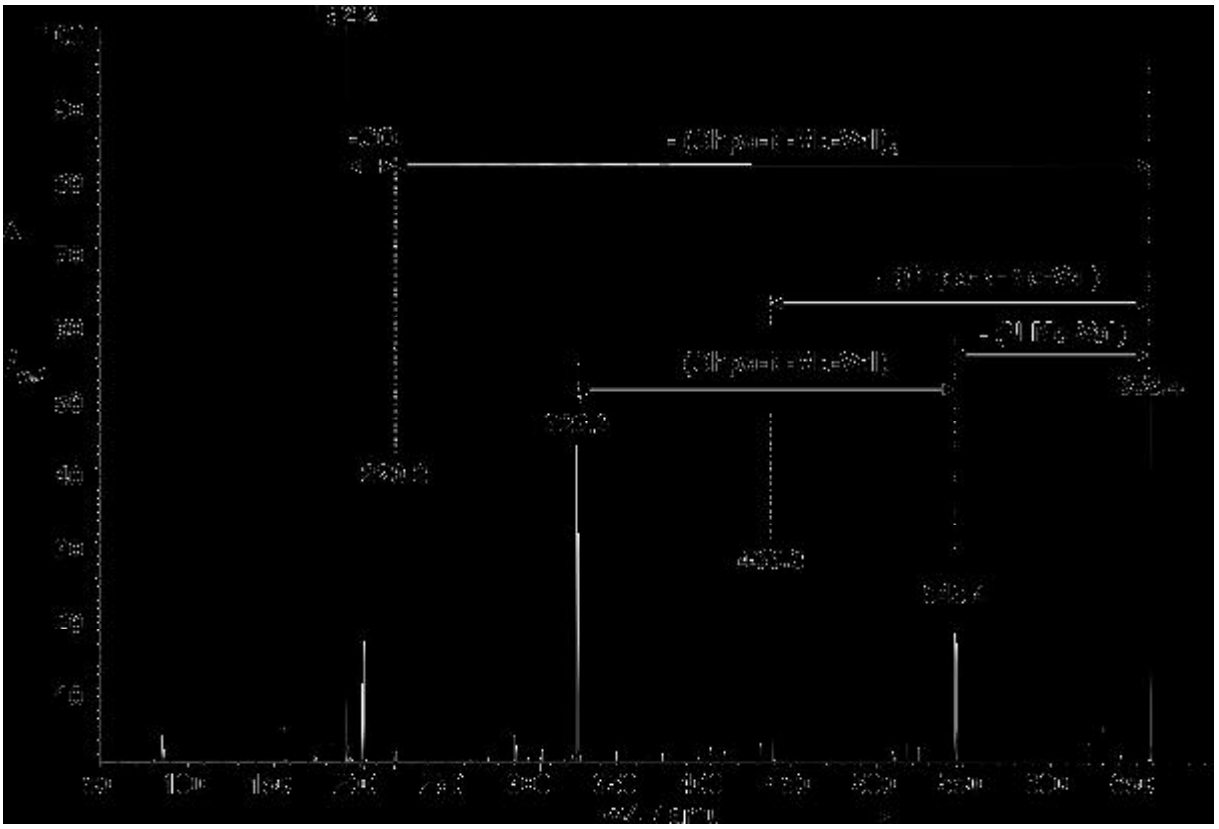
Nr.	α-D-Hydroxy acid	Structure	Incorporated into the cyclodepsi-peptide?
1	*(R)*-2-hydroxy-3-methyl-butyric acid		yes
2	*(R)*-2-hydroxy-propionic acid		yes
3	*(R)*-2-hydroxy-butyric acid		yes
4	*(R)*-2-hydroxy-pentanoic acid		yes
5	*(R)*-2-hydroxy-hexanoic acid		yes
6	*(R)*-2-hydroxy-octanoic acid		no
7	*(R)*-2-hydroxy-4-methyl-pentanoic acid		yes
8	*(R)*-2-hydroxy-3-methyl-pentanoic acid		yes
9	*(allo)*-2-hydroxy-3-methyl-pentanoic acid		yes
10	*(R)*-2-hydroxy-3,3-dimethyl-butyric acid		no

11	*(R)*-2-hydroxy-4,4-dimethyl-pentanoic acid		no
12	*(R)*-2-hydroxy-pent-4-ynoic acid		yes
13	*(R)*-2-hydroxy-4-methyl-pent-4-enoic acid		no
14	*(R)*-3-chloro-2-hydroxy-propionic acid		yes
15	*(R)*-3-fluoro-2-hydroxy-propionic acid		yes
16	*(R)*-3-bromo-2-hydroxy-propionic acid		yes
17	*(R)*-2-hydroxy-4-methylsulfayl-butyric acid		yes
18	*(R)*-3-cyclopropyl-2-hydroxy-propionic acid		yes
19	*(R)*-2,3-dihydroxy-propionic acid		no
20	*(R)*-2,3-dihydroxy-butyric acid		no
21	(*R*)-2-hydroxy-succinic acid		no

22	*(R)*-2-hydroxy-3-mercapto-propionic acid		no
23	*(R)*-3-cyclohexyl-2-hydroxy-propionic acid		no
24	*(R)*-cyclohexyl-hydroxy-acetic acid		no
25	*(R)*-hydroxy-phenyl-acetic acid		no
26	*(R)*-2-hydroxy-3-phenyl-propionic acid		no
27	*(R)*-2-hydroxy-4-phenyl-butyric acid		no
28	*(R)*-3-(4-fluoro-phenyl)-2-hydroxy-propionic acid		no
29	*(R)*-2-hydroxy-3-thiophen-2-yl-propionic acid		no
30	*(R)*-2-hydroxy-3-(1H-imidazol-4-yl)-propionic acid		no

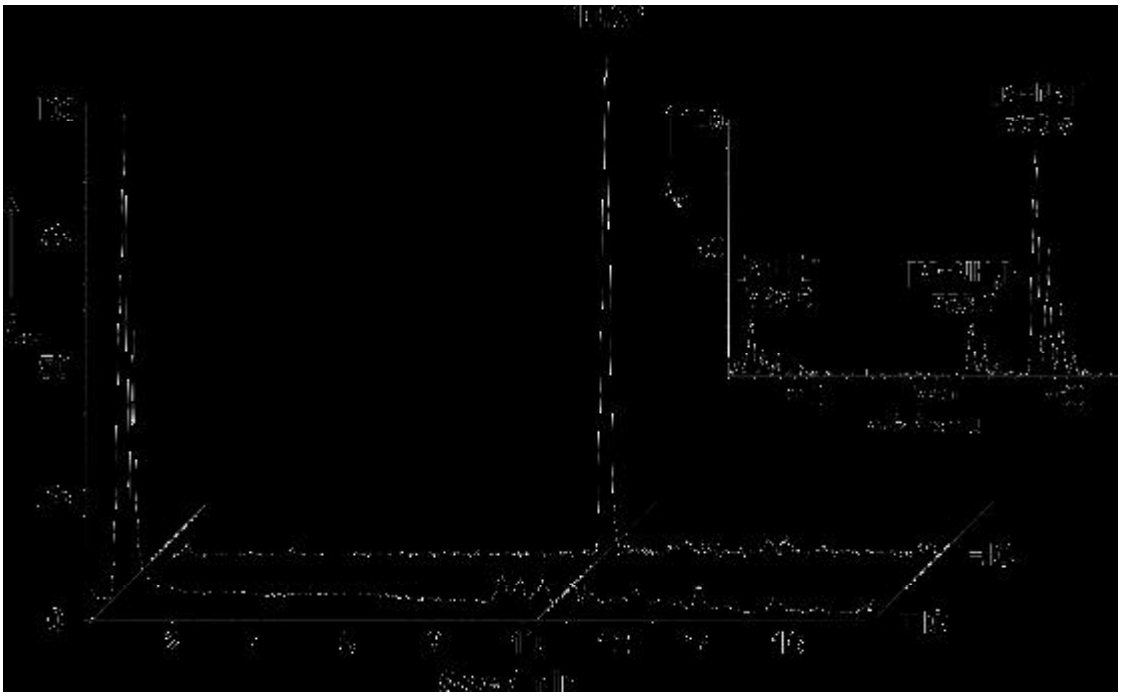
HPLC-ESI-MS and MSMS spectra:

MSMS-spectrum of 'chloro'-enniatin:

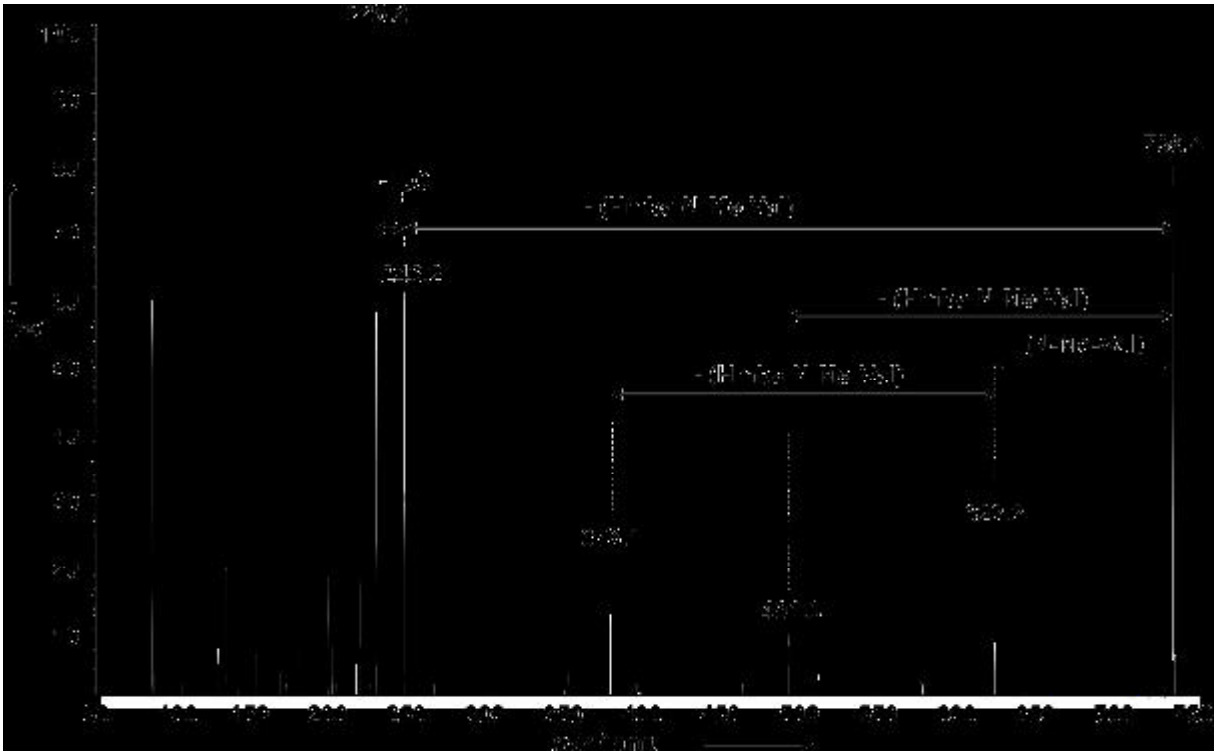


Chpa = *(R)*-3-chloro-2-hydroxy-propionic acid

HPLC-ESI-MS chromatogram of enniatin-derivative 17:



MSMS-spectrum of enniatin-derivative ***17***



Hmba = *(R)*-2-hydroxy-4-methylsulfayl-butyric acid