

Antimicrobial *N*-(2-chlorobenzyl)-substituted hydroxamate is an inhibitor of 1-deoxy-D-xylulose 5-phosphate synthase

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Electronic supplementary information

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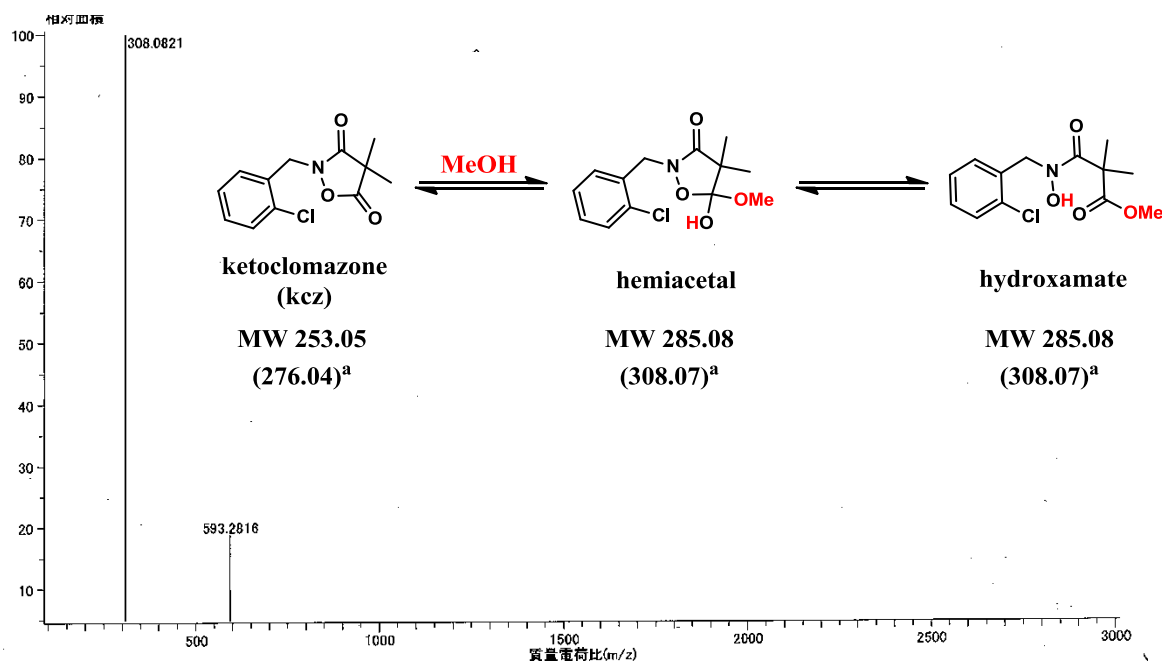


Figure S1. ESI mass spectrum of kcz in methanol solution. ^a Calculated mass for [M+Na]⁺.

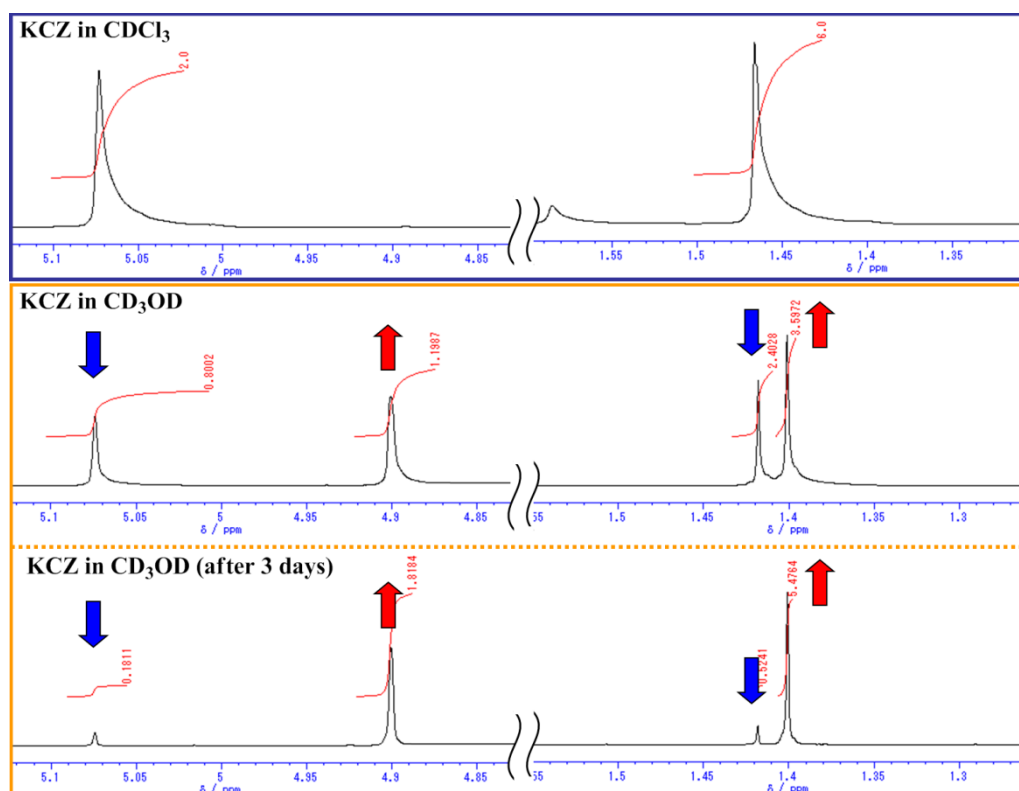


Figure S2. ¹H NMR spectra of kcz in chloroform-*d* (upper panel), and in methanol-*d*₄ at 10 min and 72 h under rt, respectively (middle and lower panel). The peaks of kcz observed in chloroform-*d* (blue) were gradually converged to a new set of singlets at 4.90 and 1.40 ppm (red), suggesting that kcz was converted to the methanol adduct.

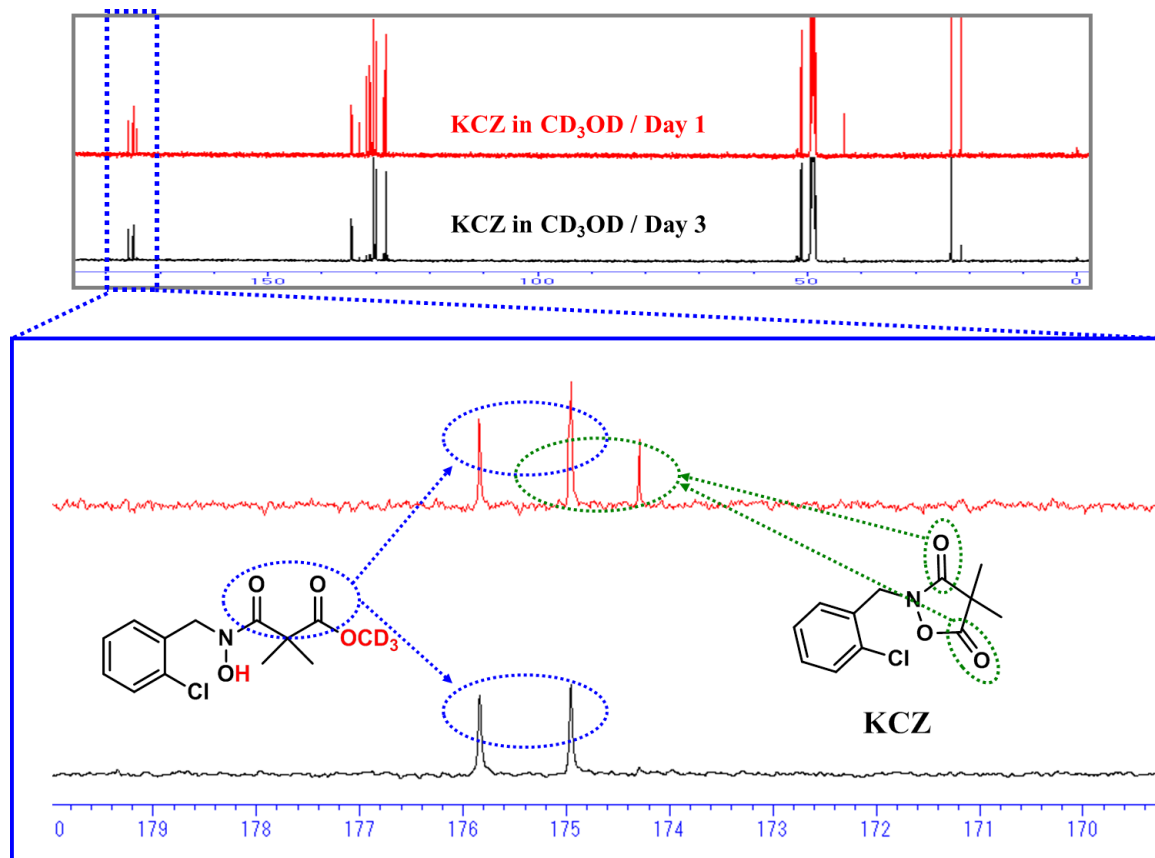
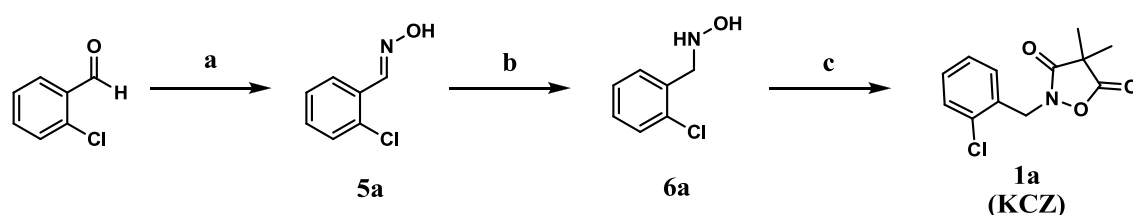


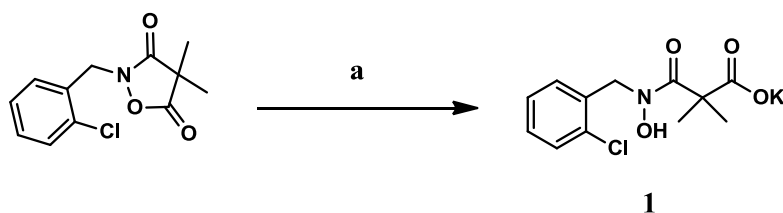
Figure S3. ^{13}C NMR spectra of kcZ in in methanol- d_4 at 10 min (red) and at 72 h (blue), respectively. Two carbonyl peaks were observed after 72 h, supporting that the resulting product is hydroxamate derivative, not hemiacetal.

General Methods. Reagents and solvents were obtained from commercial sources without further purification unless otherwise noted. ^1H and ^{13}C NMR spectra were recorded on a JEOL JNM-LA 400 spectrometer. Chemical shifts were reported in δ (ppm) relative to tetramethylsilane. All coupling constants were described in Hz. Flash column chromatography was performed on silica gel (40-63 μm) under a pressure of about 4 psi. Synthesized final compounds were checked for purity by analytical HPLC, which was performed using a JASCO PU-2086 and a JASCO UV-2075 detector with a GL Science Inertsil 150 mm x 4.6 mm, 5 μm C-18 column or TOSO TSKgel Amide-80 eluted with gradient 10% to 90 % of acetonitrile in 0.1 % TFA in water in 30 min. Elemental analysis and high-resolution mass data (HRMS) were conducted by Mr. Ttsuyoshi Matsuzaki from Comprehensive Analysis Center, ISIR, Osaka University. Preparation of ketoclofazone was carried out by a similar method to that previously reported in a literature [1].

General procedure for synthesis:



Scheme S1. Reagents and conditions for synthesis of ketoclofazone (kcz): ^a Reagents and conditions: (a) $\text{NH}_2\text{OH}\cdot\text{HCl}$, 8M NaOH, EtOH, r.t., 6 h, 72%; (b) NaBH_3CN , 2M HCl, MeOH, pH < 3, r.t., 15 h, 87%; (c) 2,2-dimethylmalonyl dichloride, TEA, toluene, r.t., 2 h, 89%.



Scheme S2. Reagents and conditions for preparation of 1: ^a Reagents and conditions: (a) 1M KOH aq. (1 eq.), THF, r.t., 2 h, 100%.

2-(2-Chlorobenzyl)-4,4-dimethyl-isoxazolidine-3,5-dione (ketoclozazone, kcZ). Preparation of ketoclozazone was carried out by a similar procedure to that previously reported in the literature [1] (Scheme S1). ^1H NMR (CDCl_3): δ 7.43-7.28 (m, 4H), 5.07 (s, 2H), 1.47 (s, 6H); ^{13}C NMR (CDCl_3): δ 173.62, 172.09, 133.74, 131.27, 130.16, 129.91, 127.20, 47.24, 41.80, 21.31; ESI-TOFMS (m/z) calcd for $\text{C}_{12}\text{H}_{13}\text{ClNO}_3$ $[\text{M}+\text{H}]^+$, 254.0578; found, 254.0575: Elemental analysis calcd (%) for $\text{C}_{12}\text{H}_{12}\text{ClNO}_3$ (253.68) C, 56.81; H, 4.77; N, 5.52; Found C, 56.55; H, 4.86; N, 5.59.

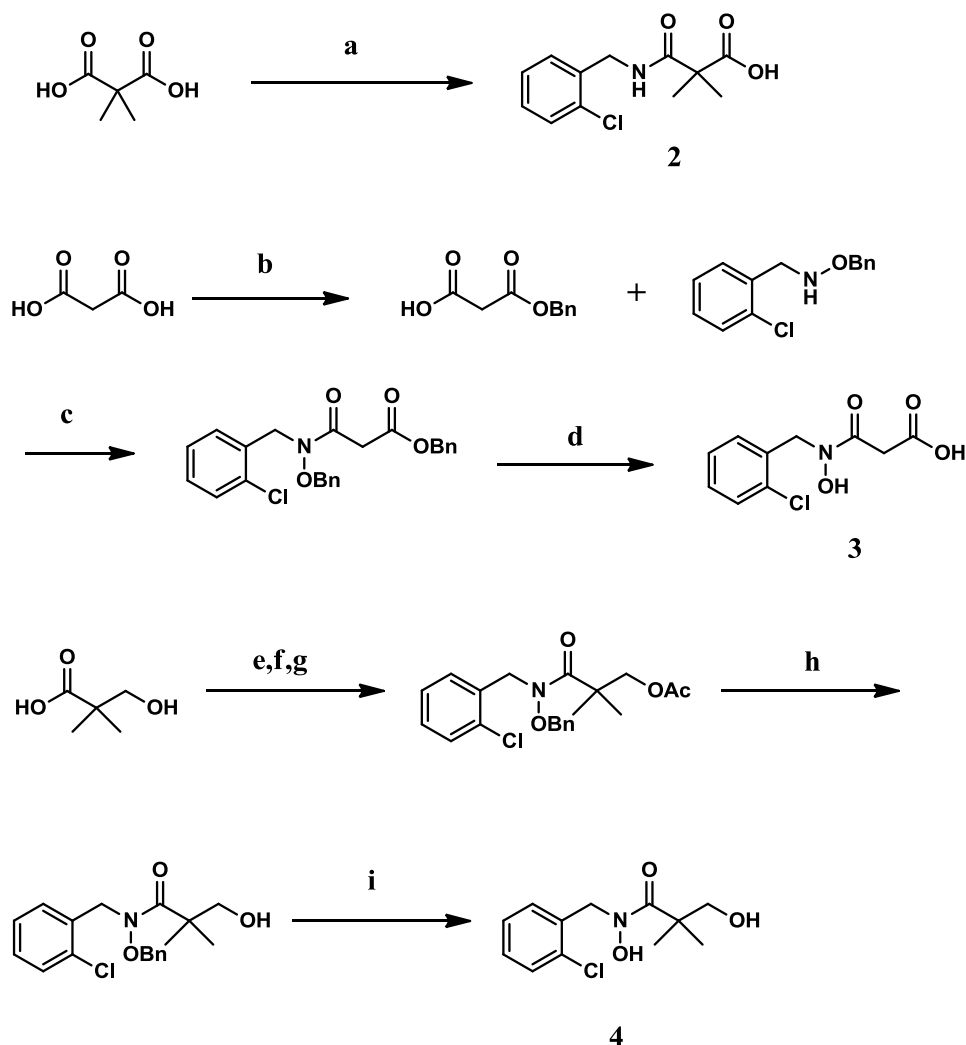
Potassium 3-((2-chlorobenzyl)(hydroxy)amino)-2,2-dimethyl -3-oxopropanoate (1). To a solution of kcZ (50 mg, 0.2 mmol) in tetrahydrofuran (2 mL) was added 1N potassium hydroxide (200 μL , 0.2 mmol) at 0 $^\circ\text{C}$, and the solution was stirred for 2h at rt. Concentration gave the product as a white solid (58 mg, 95 %). ^1H NMR (400 MHz, CD_3OD): 7.50-7.21 (m, 4H), 4.91 (s, 2H), 1.41 (s, 6H); ^{13}C NMR (100 MHz, CD_3OD): 181.83, 135.50, 134.10, 130.23, 130.20., 129.52, 128.02, 52.47, 51.56, 01.64, 23.40; ESI-TOFMS (m/z) calcd for $\text{C}_{12}\text{H}_{14}\text{ClNO}_4$ $[\text{M}+\text{H}]^+$, 272.0684; found, 272.0681: Elemental analysis calcd (%) for $\text{C}_{12}\text{H}_{13}\text{ClKNO}_3\cdot\text{H}_2\text{O}$ (327.80) C, 43.97; H, 4.61; N, 4.27; Found C, 43.60; H, 4.84; N, 4.82.

3-(2-Chlorobenzylamino)-2,2-dimethyl-3-oxopropanoic acid (2). A mixture of dimethylmalonic acid (200 mg, 1.5 mmol) and thionyl chloride (130 μl , 1.82 mmol in dichloromethane (0.4 mL) was stirred at r.t. for 2 h. To the solution was added 2-chlorobenzylamine (283 mg, 3.0 mmol) in diethylether (1.5 mL) dropwise at 0 $^\circ\text{C}$, and the mixture was stirred at r.t. for 40 min. The reaction was quenched with H_2O (20 mL), and extracted with diethylether (25 ml \times 3), washed with brine, and dried (anhyd. MgSO_4). The residual product was purified by silica gel column chromatography (hexane: ethyl acetate) to give the product as a white solid (209 mg, 51 %). ^1H NMR (CD_3OD): δ 7.36-7.14 (m, 4H), 4.47 (s, 2H), 4.47 (s, 2H), 1.46 (s, 6H); ESI-MS calcd for $\text{C}_{12}\text{H}_{14}\text{ClNNaO}_3$; 278.0650, found $[\text{M}+\text{Na}]^+$ 278.3286.

3-(Benzyloxy)-3-oxopropanoic acid. Malonic acid (208 mg, 2.0 mmol) was reacted with benzyl alcohol (414 mL, 4.00 mmol) in the presence of N, N'-dicyclohexylcarbodiimide (434 mg, 2.2 mmol) in acetonitrile (1 mL). The mixture was stirred at r.t. for 45 min, evaporated. The product was dissolved in saturated sodium bicarbonate (20 mL), and washed with ethyl acetate (20 mL x). The aqueous solution was adjusted to pH~1 with HCl, the resulting acid was extracted with dichloromethane (25 mL x3), washed with brine, and dried (anhyd. MgSO_4). Evaporation of the solvent afforded the half ester as a white solid (254 mg, 65 %). ^1H NMR (CDCl_3): δ 7.41-7.32 (m, 5H), 5.21 (s, 2H), 3.48 (s, 2H).

O-Benzyl-N-(2-chlorobenzyl)hydroxylamine. To a solution of 2-chlorobenzaldehyde (1.0 g, 7.12 mmol) and O-benzylhydroxylamine hydrochloride (1.36 g, 8.54 mmol) in ethanol (6 mL) was added 8M sodium hydroxide (1.34 mL, 10.7 mmol) dropwise at 0 $^\circ\text{C}$. The reaction mixture was stirred at r.t. overnight, and neutrized with 2N HCl. The mixture was concentrated, dissolved in dichloromethane and H_2O . The organic layer was washed with brine, dried, and the resulting residue was purified by silica gel

column chromatography (hexane: ethyl acetate) to afford 2-chlorobenzaldehyde *O*-benzyl oxime as a colorless oil (1.29 g, 74 %). $^1\text{H NMR}$ (CDCl_3): δ 8.57 (s, 1H), 7.89 (d, $J = 7.5$ Hz, 1H), 7.43-7.24 (m, 8H), 5.23 (s, 2H); $^{13}\text{C-NMR}$ (CDCl_3) δ : 146.19, 137.41, 130.89, 130.10, 129.94, 128.60, 128.54, 128.17, 127.31, 127.01, 76.82.



Scheme S3. Reagents and conditions for preparation of **2-4**: Reagents and conditions: (a) SOCl_2 (1.2 eq.), THF, reflux, 2 h, then 2-chlorobenzaldehyde (2 eq.), diethyl ether, r.t., 40 min., 50.4% (2steps); (b) BnOH (2 eq.), DCC (1.1 eq.), CH_3CN , r.t., 45 min., 65%; (c) *O*-Benzyl-*N*-(2-chlorobenzyl)hydroxylamine, EDCI, HOBt, DIEA, r.t., overnight, 46%; (d) Pd/C, H_2 , MeOH, r.t., overnight, 100%; (e) AC_2O , pyridine, r.t., overnight; (f) SOCl_2 , hexane, reflux, 5 h; (g) *O*-Benzyl-*N*-(2-chlorobenzyl)hydroxylamine, TEA, toluene, r.t., overnight, 83% (3 steps); (h) K_2CO_3 , MeOH, 0°C , 3.5 h, 49%; (i) Pd/C, H_2 , MeOH, r.t., overnight, 92%.

The oxyme (1.1 g, 4.5 mmol) was dissolved in methanol (5.5 mL), and treated with NaBCNH_3 (486 mg, 7.8 mmol). The mixture was kept under acidic condition by adding 2N HCl in methanol (~ 7 mL in

total). Solvent was evaporated, and the residual white solid was dissolved in H₂O (20 mL), of which pH was adjusted to pH ~11 with 6N NaOH. The product was extracted with dichloromethane (50 mL x 3), washed with brine, and dried (anhyd. MgSO₄). The crude product was purified by silica gel column chromatography (hexane: ethyl acetate) to give *O*-benzyl-*N*-(2-chlorobenzyl)hydroxylamine as a colorless oil (897 mg, 81 %). ¹H NMR (CDCl₃): δ 7.34-7.22 (m, 9H), 4.70 (s, 2H), 4.16 (s, 2H); ¹³C-NMR (CDCl₃) δ: 137.77, 135.05, 133.99, 131.20, 129.44, 128.76, 128.40, 128.28, 127.75, 126.68, 76.01, 53.75.

Benzyl 3-(benzyloxy(2-chlorobenzyl)amino)-3-oxopropanoate. Coupling reaction of *O*-benzyl-*N*-(2-chlorobenzyl)hydroxylamine (100 mg, 0.40 mmol) and 3-(benzyloxy)-3-oxopropanoic acid (78 mg, 0.40 mmol) was carried out with HOBt (74 mg, 0.48 mmol), DIEA (100 μL, 0.48 mmol), and EDCI (93 mg, 0.48 mmol) in DMF (4 mL). The mixture was stirred at r.t. overnight, and DMF was removed by evaporation. The residue was dissolved in ethyl acetate and H₂O, and the organic layer was washed with saturated sodium bicarbonate, brine, and dried (anhyd. MgSO₄). The crude product was purified by silica gel column chromatography (hexane : ethyl acetate), and fully protected hydroxamic acid was obtained as a colorless oil (75 mg, 44 %). ¹H NMR (CDCl₃): δ 7.38-7.19 (m, 14H), 5.13 (s, 2H), 4.75 (s, 2H), 3.53 (s, 2H).

3-((2-chlorobenzyl)(hydroxy)amino)-3-oxopropanoic acid (3). Hydrogenation of benzyl 3-(benzyloxy(2-chlorobenzyl)amino)-3-oxopropanoate (61 mg, 0.14 mmol) was carried out with 10 % Pd-C (18 mg) in methanol (2.5 mL) under atmospheric hydrogen. The mixture was stirred overnight, filtered, then concentrated to give the product as colorless oil (35 mg, 100 %). ¹H NMR (CD₃OD): δ 7.32 (s, 5H), 4.77 (s, 2H), 3.55 (s, 2H); ESI-TOFMS (*m/z*) calcd for C₁₀H₁₁ClNO₄ [M+H]⁺, 244.0371; found, 244.1191.

3-(Benzyloxy(2-chlorobenzyl)amino)-2,2-dimethyl-3-oxopropyl acetate. A solution of hydroxypivalic acid (185 mg, 1.56 mmol) and acetic anhydride (294 μL, 3.12 mmol) in pyridine (1 mL) was stirred at r.t. overnight, and concentrated. The resulting acetylated pivalic acid was dissolved in hexane (3 mL) and catalytic amount of DMF and oxalyl chloride (268 μL, 3.12 mmol) was added, and the mixture was then refluxed for 5 h. After concentration, the resulting acetyl protected acid chloride was dissolved in toluene (2 mL), and added to a solution of *O*-benzyl-*N*-(2-chlorobenzyl)hydroxylamine (50 mg, 0.20 mmol) and triethylamine (56 μL, 0.40 mmol) in toluene (2 mL) at 0 °C. The reaction mixture was then stirred at r.t. overnight, quenched with H₂O, and extracted with ethyl acetate (20 mL). The organic layer was washed with saturated sodium bicarbonate, brine, and dried (anhyd. MgSO₄). The crude material was purified by silica gel column chromatography (hexane : ethyl acetate) to afford 3-(benzyloxy(2-chlorobenzyl)amino)-2,2-dimethyl-3-oxopropyl acetate as a colorless oil (65 mg, 83 %). ¹H-NMR (CDCl₃) δ: 7.41-7.22 (m, 9H), 5.12 (s, 2H), 4.86 (s, 2H), 4.25 (s, 2H), 2.07 (s, 3H), 1.32 (s, 6H).

***N*-(Benzyloxy)-*N*-(2-chlorobenzyl)-3-hydroxy-2,2-dimethylpropanamide.** Acetyl group of 3-(benzyloxy(2-chlorobenzyl)amino)-2,2-dimethyl-3-oxopropyl acetate (30 mg, 0.078 mmol) as removed

by treatment with potassium carbonate (22 mg, 0.156 mmol) in methanol (1 mL). The solution was stirred at r.t. for 1.5 h, and concentrated. The residue was dissolved in H₂O, and pH was adjusted to ~7 with 2N HCl. The product was extracted with chloroform, washed with brine, and dried (anhyd. MgSO₄). The crude product was purified by silica gel column chromatography (hexane: ethyl acetate = 2:1) to afford free alcohol as a colorless oil (6 mg, 22 %). ¹H-NMR (CDCl₃) δ: 7.39-7.21 (m, 10H), 5.09 (s, 2H), 4.90 (s, 2H), 3.57 (s, 2H), 3.15 (s, 1H), 1.32 (s, 6H).

N-(2-Chlorobenzyl)-N,3-dihydroxy-2,2-dimethylpropanamide (4). A solution of *N*-(benzyloxy)-*N*-(2-chlorobenzyl)-3-hydroxy-2,2-dimethylpropanamide (6 mg, 0.017 mmol) and 10 % Pd-C (1.8 mg) in methanol (2 mL) was stirred under atmospheric hydrogen overnight. The reaction mixture was filtered, and concentrated to give the product (4 mg, 92 %). ¹H-NMR (CD₃OD) δ: 7.34-7.23 (m, 4H), 4.77 (s, 2H), 3.67 (s, 2H), 1.27 (s, 6H); ESI-TOFMS (*m/z*) calcd for C₁₂H₁₇ClNO₃ [M+H]⁺, 258.0897; found, 258.1343.

Expression and purification of recombinant *H. influenzae* DXS (HiDXS):

Recombinant *Haemophilus influenzae* DXS synthase (HiDXS) was expressed and purified by a procedure reported in literature [2], and stored at -85 °C. Purified protein was checked by SDS-PAGE (Fig. S4).

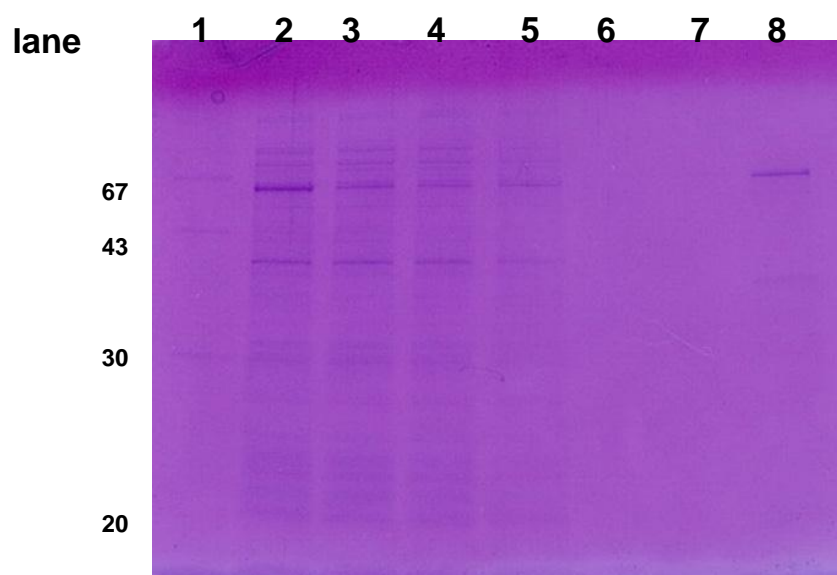


Figure S4. SDS-PAGE of column fractions of HiDXS: lane: 1, Mw marker; 2, HiDXS crude extract; 3, supernatant; 4, free flow; 5, 60 mM imidazole; 6, 250 mM imidazole washed; 7, after dialysis; 8, HiDXS concentrated.

Inhibition assay for HiDXS coupled with NADH-driven LDH reaction:

The first HiDXS assay was performed by the procedure to that previously reported by Kuzuyama et al [1], except that in this study we used D-(+)-glyceraldehyde (D-GA, $K_m = 5.6$ mM) [3] instead of DL-GAP [1]. Following LDH/NADH assay for determining the amount of remaining pyruvate was carried out under the condition described below. Prior to running assay, following buffer solutions as well as stock solution of reagents were prepared: assay buffer (40 mM Tris HCl, pH 7.61, 100 mM NaCl, 1 mM DDT, 1mM $MgCl_2$; DTT was added immediately before the experiment), thiamine pyrophosphate chloride (TPP, MP Biomedicals, 30 mM in H_2O), D-(+)-glyceraldehyde (GA, Sigma, 2M in DMSO), NADH (Roche, 7 mM in 5% $NaHCO_3$), 75 ku/L L-lactate dehydrogenase (LDH, Sigma, 6760 ku/L, this should be diluted with assay buffer immediately prior to use), and pyruvic acid (WAKO, 12 mM in H_2O). Pyruvate solution was prepared immediately prior to the experiment. Stock solutions of each compound (10 mg / mL) were prepared with DMSO (kcz, 2) or H_2O (1, 3, 4).

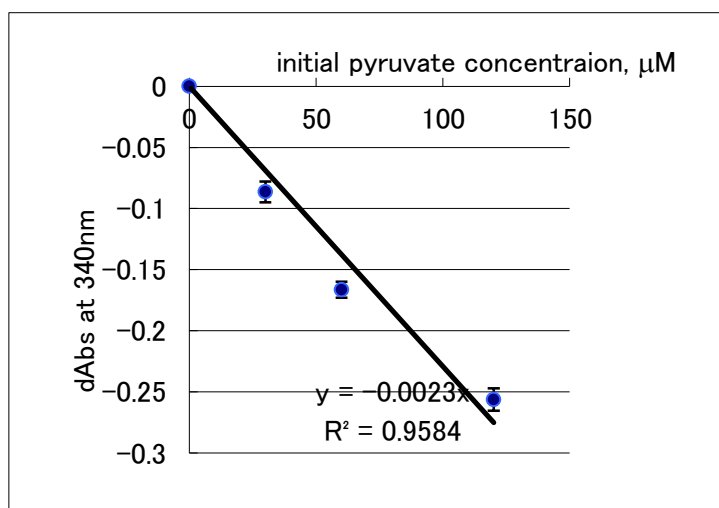


Figure S5. Initial concentration of pyruvate used for DXS assay is well correlated with the amount of NADH consumed for LDH reaction. This experiment was performed by the procedure described above, except for using buffer to replace solutions of compound and HiDXS. Each experiment was performed in duplicate.

Procedure for testing various compounds at 12.8 $\mu g / mL$. In each well of 96 well plate (Greiner) was placed 3.2 μL of 1 mg / mL compound solution in DMSO, and 2.5 μL of 12 mM pyruvate stock solution, which was distributed by a multichannel pipetter. To each well, 239.3 μL of assay buffer containing 20 mM GA and 0.3 mM TPP was added by a multichannel pipetter. Finally, 5 μL of HiDXS solution (30 pmol) [4] was added to each well (final volume = 250 μL , [GA] = 19.1 mM, [TPP] = 0.29 mM, [pyruvate] = 120 μM , compound = 12.8 $\mu g / mL$). The plate was rapped with plastic, and incubated

at 37 °C for 2 h (TITEC plate shaker, 400 rpm). After incubation, 200 µL of each reaction mixture was transferred by multichannel pipetter to another fresh well, in which 10 µL of 7 mM NADH and 6 µL of 75 ku/L LDH were placed beforehand ([NADH] = 324 µM, LDH = 2.08 ku / L). The plate was stood at r.t. for 10 min, and absorbance at 340 nm was measured by microplate reader (Spectramax M5, Molecular Devices). Inhibition activity against HiDXS was calculated by relative amount of remained pyruvate compared to controls.

Growth inhibition assay against *H. influenzae*:

Primary culturing *H. influenzae* ATCC43095 was carried out following the procedure provided by ATCC (#43095). Cells were plated on Chocolate plate (ATCC #814 agar medium), and the plates were incubated at 35 °C in an atmosphere of 5 % for 20 h. Well-separated colonies were inoculated in a liquid medium (ATTC#5129): bovin hematin (Sigma-Aldrich, 15.0 mg / L), Mueller Hinton Broth (Difco 275730; 21.0 g / L), NAD (15.0 mg / L), yeast extract (5.0 g / L), MgCl₂ (100 mg / L), CaCl₂ (100 mg / L), and pH was adjusted to 7.3. Optimal density at 570 nm was adjusted to 0.5, and the cell stock solution was diluted to 1:600 with the liquid medium. Aliquots (154.4 µL) of the cell stock solution were placed into each well of a 96-well plate. To each well, compound solution (25.6 µL; 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128 µg / µL), and DX solution (20 µL of 10 mg / mL, 200 µg) or H₂O (20 µL) were added. The plate was incubated at 35 °C for 20 h. MIC values were read as the lowest concentration with no visible growth.

References:

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