Reductive Biotransformation of Nitroalkenes via Nitroso-intermediates to Oxazetes Catalyzed by Xenobiotic Reductase A (XenA)

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Source of enzymes, compounds and materials

TLC were run on silica (Merck silica gel 60, F_{254}). IR spectra were recorded as film on NaCl plates on a Unicam Galaxy 7020 FTIR spectrometer. All NMR spectra were acquired at 25 °C on a Bruker Avance III 300 MHz NMR spectrometer, equipped with a 5mm TXI probe and z-axis gradients. For the two-dimensional NMR spectra (HSQC and HMBC), data matrices of 2048x256 (F2xF1) points were acquired with 4 and 16 scans for the HSQC and HMBC spectrum, respectively. Chemical shifts are reported in ppm [δ] and coupling constants are given in Hz. High Resolution MS measurements were performed with Narrow SIM @ 1.250.000 resolution FWHH. Differential scanning calorimetric measurement were carried out using a Simultaneous Thermoanalysator (STA 449 C, Netzsch) using helium as carrier gas. 2-Phenyl-1-propene, acetic anhydride, nitric acid, acetophenone (**1f**), 2-phenylpropanal (**1c**), 1-nitrocyclohexene (**2**), (*E*)-1-nitro-2-phenyl-ethene (**4**), (*E*)-2-nitro-1-phenyl-1-propene (**3**), propiophenone (**5f**, 95%) and methyltriphenylphosphonium bromide (97%) were from Aldrich. Nitromethane for synthesis was from Merck and ether (99.5%) extra dry over molecular sieves was from Acros Organics. NADPH and $NADP^+$ were purchased from Biocatalytics (Order number 004642), NADH, NAD^+, glucose dehydrogenase (475 U/ml) were from Codexis. The plasmids for pentaerythritol tetranitrate reductase from *Enterobacter cloacae* PB2 (PETN-Red, 2.6 mg/ml) and morphinone reductase from *Pseudomonas putida* M10 (Mor-Red, 1.9 mg/ml) were provided by Neil C. Bruce (Department of Biology, University of York), enzyme production was performed as recently reported.[1] *Lycopersicon esculentum* OPR3 was expressed and purified as recently reported.[2] YhdA from *Bacillus subtilis* was expressed and purified using a standard protocol.[3]

Synthesis of substrates and reference materials

(*E*/*Z*)-1-Nitro-2-phenylprop-1-ene (1): To a stirred mixture of acetic anhydride (40ml) and nitric acid (5.28 g, 65%) was added 2-phenylpropene (3.2 ml, 12.2 mmol) at 0 °C. After 20 min, the solution was poured into water (180ml) and stirred for additional 30 min. The organic layer was washed with saturated aqueous $NaHCO_3$, water and then dried (Na_2SO_4). Removal of the solvent under reduced pressure gave an oily residue of crude 2-acetoxy-1-nitro-2-phenylpropane, that was purified via flash chromatography on silica (ethyl acetate/petroleum ether 1:30). A solution of the nitroacetate in triethylamine (15ml) and chloroform (30ml) was stirred for 3h at room temperature. After the addition of HCl (2N,

30ml), the mixture was extracted with dichloromethane and dried (Na_2SO_4). Evaporation of the solvent followed by flash chromatography on silica (ethyl acetate/petroleum ether 1:25) afforded 1-nitro-2-phenylprop-1-ene (*E*/*Z*)-**1** as yellow oil (788 mg, 5.97 mmol, 49%).[4] GC-MS (EI): *m/z* 39, 44, 51, 65, 77, 91, 115, 120, 130, 135, 145, 163; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 2.66 (3H, d, J=1.3); 7.32 (1H, d, J=1.4); 7.46 (5H, s). NMR data corresponded to literature.[5,6,7]

1-Nitro-2-phenylpropane (1a): *trans*-β-Nitrostyrene (0.45 g, 3 mmol) in 20ml dry Et_2O was added to methyl magnesium iodide (5ml of a 3M solution, 15 mmol) in 40ml of dry Et_2O at -20 °C. Within 10 min, the solution was added to ice cold 5% aqueous HCl solution and stirred for 30 min. The mixture was extracted with CH_2Cl_2, dried over Na_2SO_4, filtered and the solvent was evaporated to give 1-nitro-2-phenylpropane **1a** (124 mg, 0.75 mmol, 25%).[2] ^{1}H-NMR ($CDCl_3$): δ 1.4 (3H, d, J=7.0), 3.62-3.72 (1H, m), 4.47-4.60 (2H, m), 7.23-7.38 (5H, m); ^{13}C-NMR ($CDCl_3$): δ 18.7, 38.6, 81.9, 126.9, 127.6, 129.0, 140.9. NMR data corresponded to literature.[4,5,6]

(*E/Z*)-2-Phenyl-propanaloxime (1b): To a stirred solution of 2-phenylpropanal **1c** (200 µL, 1.79 mmol) in 5 ml of ethanol, hydroxylamine hydrochloride (249 mg, 3.58 mmol) was added together with pyridine (435 µL, 5.37 mmol). After 24h the mixture was washed with water (20ml) and extracted twice with ethyl acetate. The combined organic layers were washed with brine and dried over Na_2SO_4 and evaporated. The product was purified by flash chromatography on silica (ethyl acetate/petroleum ether 1:5) to give **1b** as a liquid (116 µL, 1.04 mmol, 58%).[8] GC-MS (EI): *m/z* 39, 51, 63, 77, 91, 105, 117, 132, 149; ^{1}H-NMR ($CDCl_3$): δ 1.46-1.51 (3H, d, J=6.5), 3.67-3.76 (1H, m), 4.45-4.52 (1H, m), 7.26-7.40 (5H, m), 7.55-7.58 (1H, d, J=7.2), 8.21 (OH s, br); ^{13}C-NMR ($CDCl_3$): δ 18.3, 18.8, 35.0, 40.4, 126.8, 127.0, 127.2, 127.4, 128.7, 128.8, 141.9, 142.1, 141.9, 142.1, 154.8, 155.3. NMR data corresponded to literature.[9]

2-Phenyl-1-butene (starting material for **5**)**:** In a two-necked round-bottomed flask under argon, a solution of Ph_3PMeBr (0.91 equiv, 1.42 g, 4.55 mmol) and *n*-BuLi (0.91equiv, 1.82ml of 2.5M solution, 4.55 mmol) in Et_2O (0.34M, 11.38ml) was prepared. After stirring the reaction mixture for 4h at 0 °C, a solution of propiophenone **5f** [1 equiv, 671 mg, 65 mmol in Et_2O (2.2M, 2.28ml)] was added. The reaction mixture was stirred at reflux overnight and filtered. The resulting solution was poured into water and extracted with Et_2O (3x). The combined organic phases were dried over $MgSO_4$, concentrated in vacuum and the crude

material was purified by flash chromatography (ethyl acetate/petroleum ether 1:100) to afford pure 2-phenyl-1-butene (510 mg, 3.86 mmol, 76%). ^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.12-1.17 (3H, t, J=7.4); 2.52-2.60 (2H, q, J=7.2); 5.10 (2H, s), 5.32 (2H, s), 7.28-7.47 (Ar, m); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 13.0, 28.1, 111.0, 126.0, 127.3, 128.2, 141.6, 150.1. NMR data corresponded to literature.[10]

(*E/Z*)-1-Nitro-2-phenylbut-1-ene (5): To a stirred mixture of acetic anhydride (7ml) and 60% nitric acid (1g) was added 2-phenyl-butene (500 mg, 3.79 mmol) at 0 °C. After 20 min, the solution was poured into water (30ml) and stirred for additional 30 min. The organic layer was washed with aqueous $NaHCO_3$, water and dried. Removal of the solvent under reduced pressure gave 2-acetoxy-1-nitro-2-phenylbutane, which was used without purification. It is important to note that for the elimination of acetic acid from 2-acetoxy-1-nitro-2-phenylbutane yielding 1-nitro-2-phenyl-1-butene **5** (instead of isomeric 1-nitro-2-phenyl-2-butene) it was necessary to use saturated $NaHCO_3$ solution instead of Et_3N in chloroform. A solution of the nitroacetate in saturated $NaHCO_3$ (2 ml) and chloroform (4 ml) was stirred overnight. After the addition of HCl (2N), the mixture was extracted with dichloromethane and dried (Na_2SO_4). Evaporation of the solvent followed by flash chromatography on silica (ethyl acetate/petroleum ether 1:10) afforded 1-nitro-2-phenylbut-1-ene **5** as yellow oil (103 mg, 0.58 mmol, 21%). GC-MS (EI): *m/z* (one isomer) 30, 39, 51, 65, 77, 91, 103, 115, 131, 147, 159, 177; (one isomer) 32, 43, 51, 65, 77, 91, 105, 115, 124, 133, 145, 160, 177; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.15-1.20 (3H, t, J=7.4), 3.07-3.15 (2H, q, J=7.5), 7.21 (1H, s, br); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 12.4, 12.8, 24.8, 29.6, 126.4, 127.1, 127.8, 128.5, 129.0, 130.2, 135.7, 137.1, 155.8. Overall, NMR data corresponded to literature.[4,10]

(*E/Z*)-1-(2´-Naphthyl)-1-nitro-1-propene (6): In a 100ml flask under argon was prepared a solution of 1-(2-naphthyl)-ethanone **6f** (2.66 g, 12.5 mmol, 1equiv), 2.68ml nitromethane (50 mmol, 4 equiv) and 498µl of *n*-$BuNH_2$ (5 mmol, 0.4equiv) in toluene (8ml). The resulting mixture was stirred under reflux overnight with a Dean-Stark apparatus. It was then cooled to room temperature, diluted with EtOAc and quenched by addition of an aqueous solution of NH_4Cl. The organic phase was separated, dried over $MgSO_4$ and concentrated under reduced pressure to afford the crude product, which was purified by flash chromatography on silica gel (ethyl acetate/petroleum ether 1:100) afforded (*E/Z*)-1-(2´-naphthyl)-1-nitro-1-propene **6** (1.45 g, 55%). GC-MS (EI): *m/z* (one isomer) 30, 39, 49, 58, 70, 83, 91, 101, 115, 128, 139, 152, 165, 183, 195, 213; (one isomer) 39, 63, 82, 98, 115, 141, 165, 196, 213; ^{1}H-NMR (300

MHz, $CDCl_3$): δ 2.17 (3H, s), 2.82 (3H, s), 7.28 (1H, =CH-NO_2, s), 7.47- 7.97 (Ar, m); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 18.6, 31.0, 123.7, 126.7, 127.1, 127.5, 127.7, 128.6, 128.9, 133.0, 134.0, 135.4, 136.6, 149.9. NMR data deviated in part from literature.[10]

Analytical procedures

GC-MS measurements were carried out with an Agilent 7890A GC system, equipped with an Agilent 5975C mass-selective detector (electron impact, 70eV) using an Agilent J&W Scientific HP-5MS column (5% phenyl-methyl-polysiloxane, length 30m, I.D. 250µm, film 0.25µm). GC-FID analyses were carried out on a Varian 3800 gas chromatograph using H_2 as carrier gas (14.5psi).

Determination of conversion

Conversions of (*E/Z*)-1-nitro-2-phenyl-1-propene **1**, 1-nitrocyclohexene **2**, (*E*)-2-nitro-1-phenyl-1-propene **3,** (*E*)-1-nitro-2-phenyl-ethene **4** and (*E/Z*)-1-nitro-2-phenylbut-1-ene **5** were analyzed using a 6% cyanopropyl-phenyl phase capillary column (Varian CB 1701, 30m, 0.25mm, 0.25µm), detector temperature 240 °C. Temperature program 70 °C hold 0 min, 5 °C/min to 140 °C, hold 0 min, 25 °C/min to 170 °C, hold 15 min.

Retention times for the bioreduction products of (*E/Z*)-1-nitro-2-phenyl-1-propene **1**: acetophenone **1f** 5.95 min, 2-phenyl-1-propanal **1c** 6.54 min, (*E/Z*)-2-phenyl-propan-1-oxime **1b** 12.48 and 13.06 min, 1-nitro-2-phenyl-propane **1a** 13.69 min, (*E*/*Z*)-1-nitro-2-phenyl-propene **1** 14.84 min and 15.44 min, 4-methyl-4-phenyl-4*H*-1,2-oxazete **1e** 16.48 min. Retention times for the bioreduction products of (*E/Z*)-1-nitro-2-phenylbut-1-ene **5**: phenylpropanone **5f** 9.04 min, 1-nitro-2-phenyl-butane **5a** 13.80 min, (*E/Z*)-1-nitro-2-phenylbut-1-ene **5** 16.04 min and 16.82 min, 4-ethyl-4-phenyl-4*H*-1,2-oxazete **5e** 18.95 min; Retention times for the bioreduction products of 1-nitrocyclohexene **2**: cyclohexanone **2c** 3.15 min, nitrocyclohexane **2a** 7.41 min, 1-nitrocyclohexene **2** 9.7 min; Retention times for the bioreduction products of (*E*)-1-nitro-2-phenyl-ethene **4**: 1-nitro-2-phenyl-ethane **4a** 13.7 min, (*E*)-nitro-2-phenyl-ethene **4** 15.61 min; Retention times for the bioreduction products of (*E*)-2-nitro-1-phenyl-1-propene **3**: 1-phenylpropan-2-one **3c** 8.27 min, 2-nitro-1-phenyl-propane **3a** 14.08 min, (*E*)-2-nitro-1-phenyl-1-propene **3** 16.17 min. Conversion of (*E/Z*)-1-(2´-naphthyl)-1-nitro-1-propene **6** was analyzed using a HP-5MS column (5% phenyl-methyl-polysiloxane, length 30m, I.D. 250µm, film 0.25µm). Temperature program: 100 °C hold 0.5 min, 10 °C/min to 300 °C, hold 0 min. Retention times for the bioreduction products of **6**: 2-acetonaphthone/ 1-(2´-naphtyl)-1-ethanone **6f** 10.70 min, 2-(1-nitropropan-2-yl)naphthalene

6a 13.79 min, (*E/Z*)-1-(2´-naphthyl)-1-nitro-1-propene **6** 14.27 min and 15.05 min, 4-methyl-4-(naphthalen-2-yl)-4*H*-1,2-oxazete **6e** 14.86 min.

Determination of enantiomeric excess of 1e

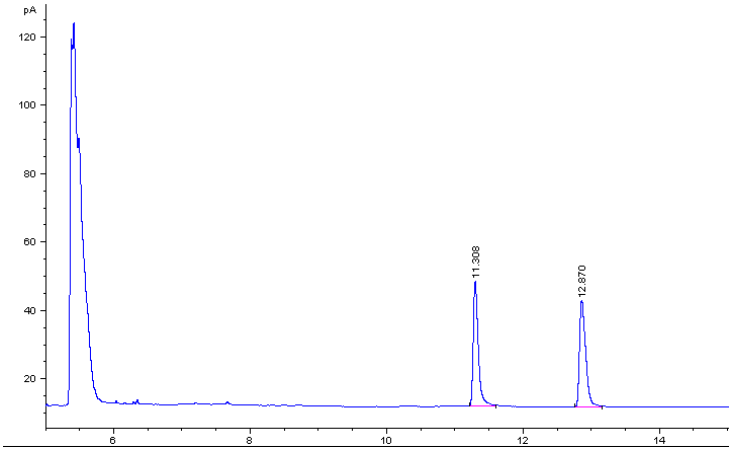


Figure S1 Chiral GC measurement of **1e**.

The enantiomeric excess of 4-methyl-4-phenyl-4*H*-1,2-oxazete **1e** was determined using a CP-Chirasil-DEX CB (25m, 0.32mm, 0.25µm film). Temperature program for **1e**: 110 °C hold 1 min, 15 °C/min to 170 °C, hold 15 min, 10 °C/min to 180 °C, hold 0 min. Retention times: *(S/R)* or *(R/S)*-**1e** 11.31 min and 12.87 min. No reference material was available.

Differential Scanning Calorimetry (DSC)

5.001 mg of 4-methyl-4-phenyl-4*H*-1,2-oxazete **1e** was weighed into a special aluminum vessel and aligned. The measurement was performed on a Simultaneous Thermoanalysator (STA 449 C, Netzsch) with helium as carrier gas (50 mL/min). The heating rate was 10 °C/min.

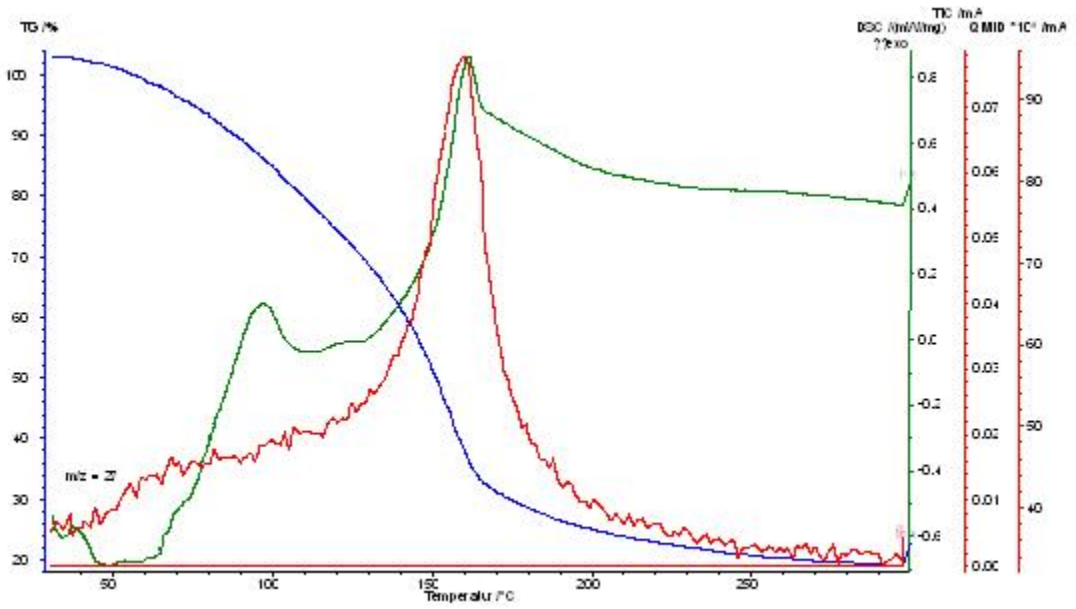
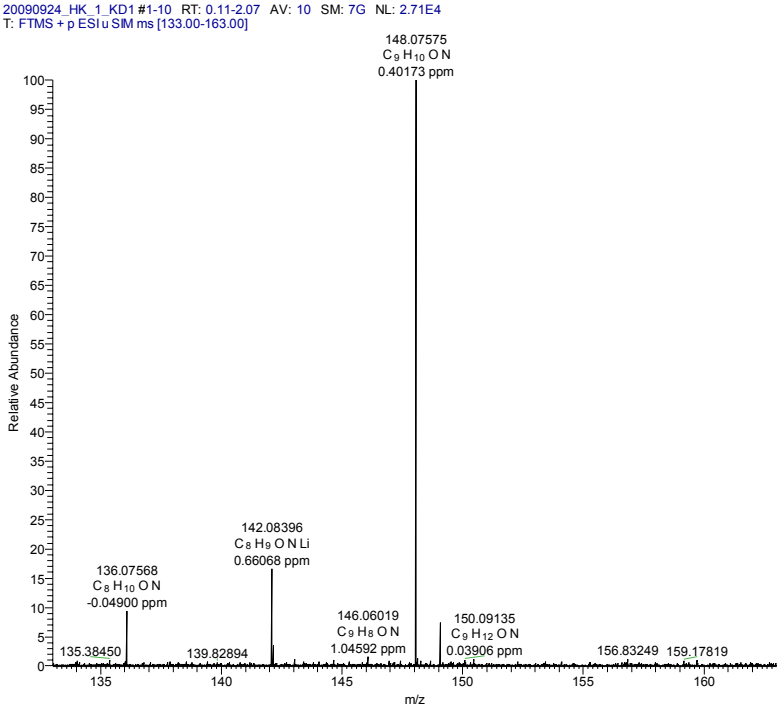
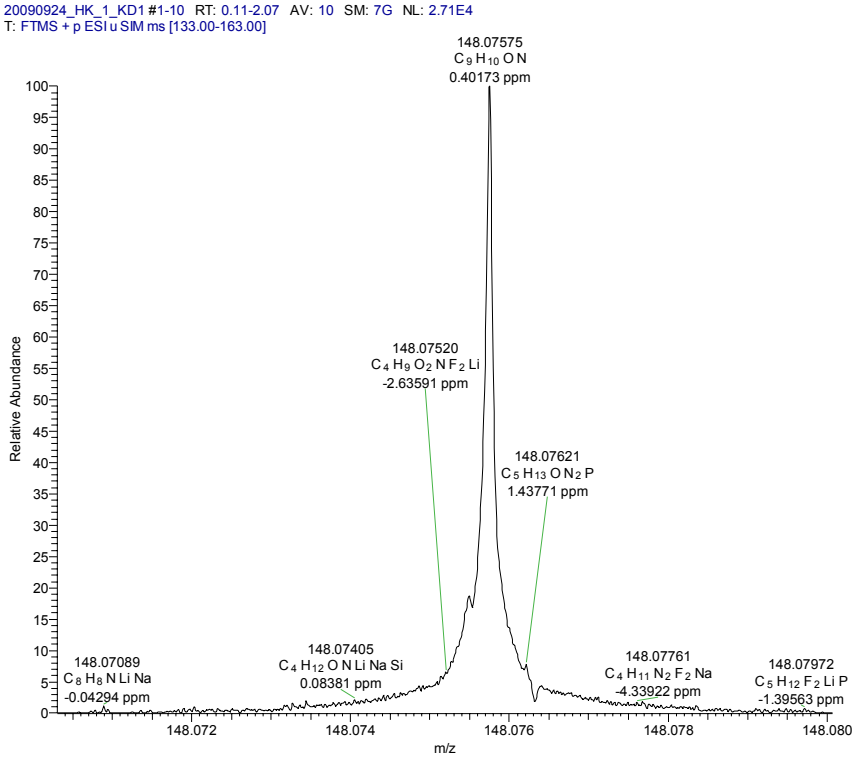


Figure S2 DSC measurement of **1e:** X-axis: temperature range; y-axis decreasing mass / weight percentage of 4-methyl-4-phenyl-4*H*-1,2-oxazete **1e**. Green line: heat tone; blue line: mass percentage of **1e**; red line: mass to charge ratio of HCN (27), indicating increased HCN-formation within the temperature range of 120-160 °C with concomitant decreasing mass percentage of **1e**.

High Resolution MS (HRMS)

The sample was prepared by dissolving 15 mg of oxazete **1e** in 1ml MeOH, measurements were performed on a Narrow SIM @ 1.250.000 resolution FWHH MS





Rank	Elemental composition	RDB	ppm
1	$C_9H_{10}ON$	5.5	0.40182
2	$C_4H_9O_2NF_2Li$	-0.5	1.07735
3	$C_5H_{13}ON_2P$	1	-1.69569
4	$C_6H_{11}ONLiSi$	2.5	-4.7005

Figure S3 High resolution mass spectra of **1e**

NMR Spectra

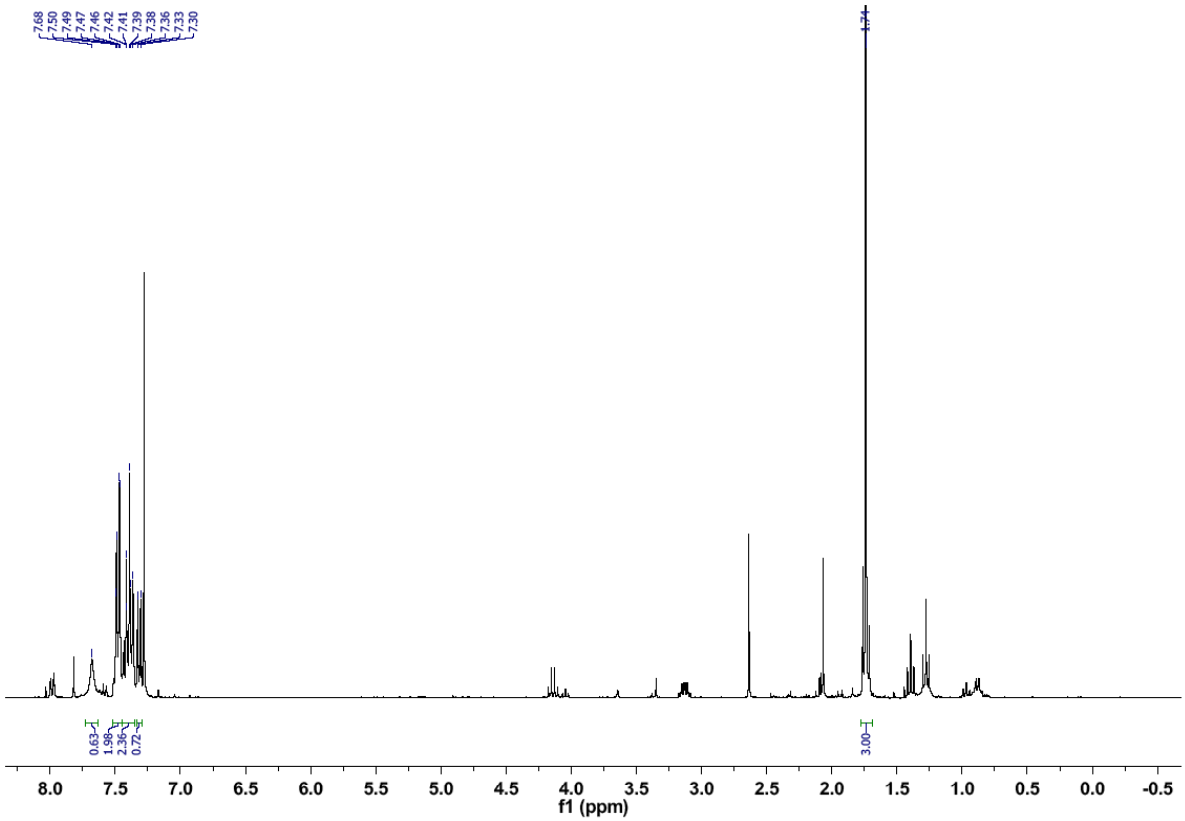


Figure S4 ^{1}H-NMR of oxazete **1e**

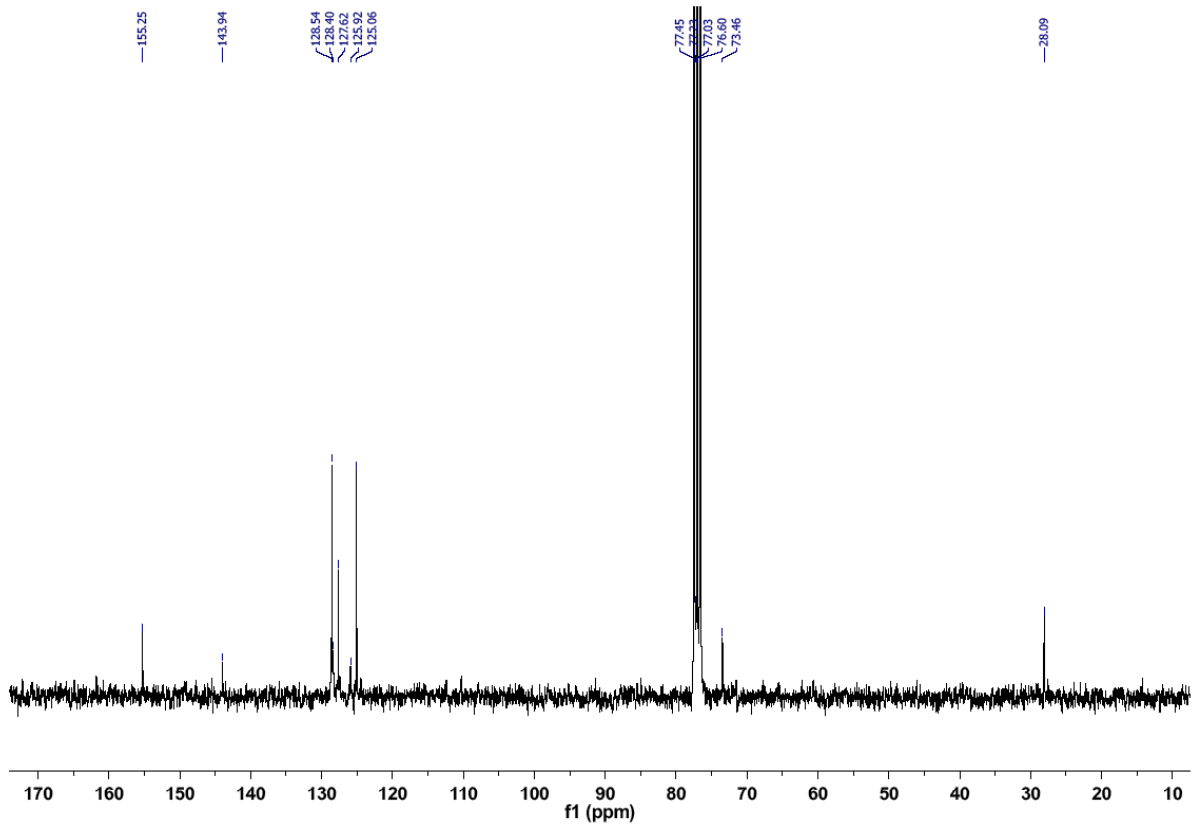


Figure S5 ^{13}C-NMR of oxazete **1e**

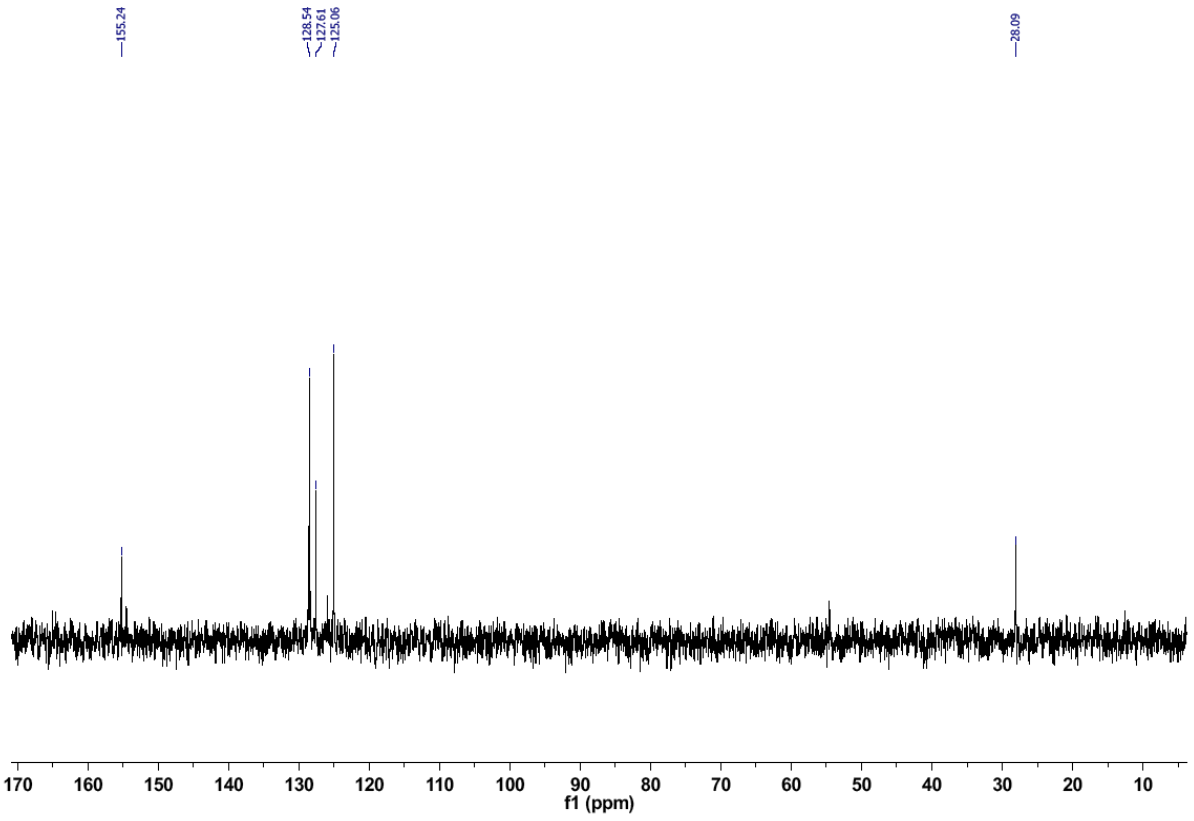


Figure S6 135-DEPT ^{13}C-NMR of oxazete **1e** (CH and CH_3 positive, CH_2 negative signals)

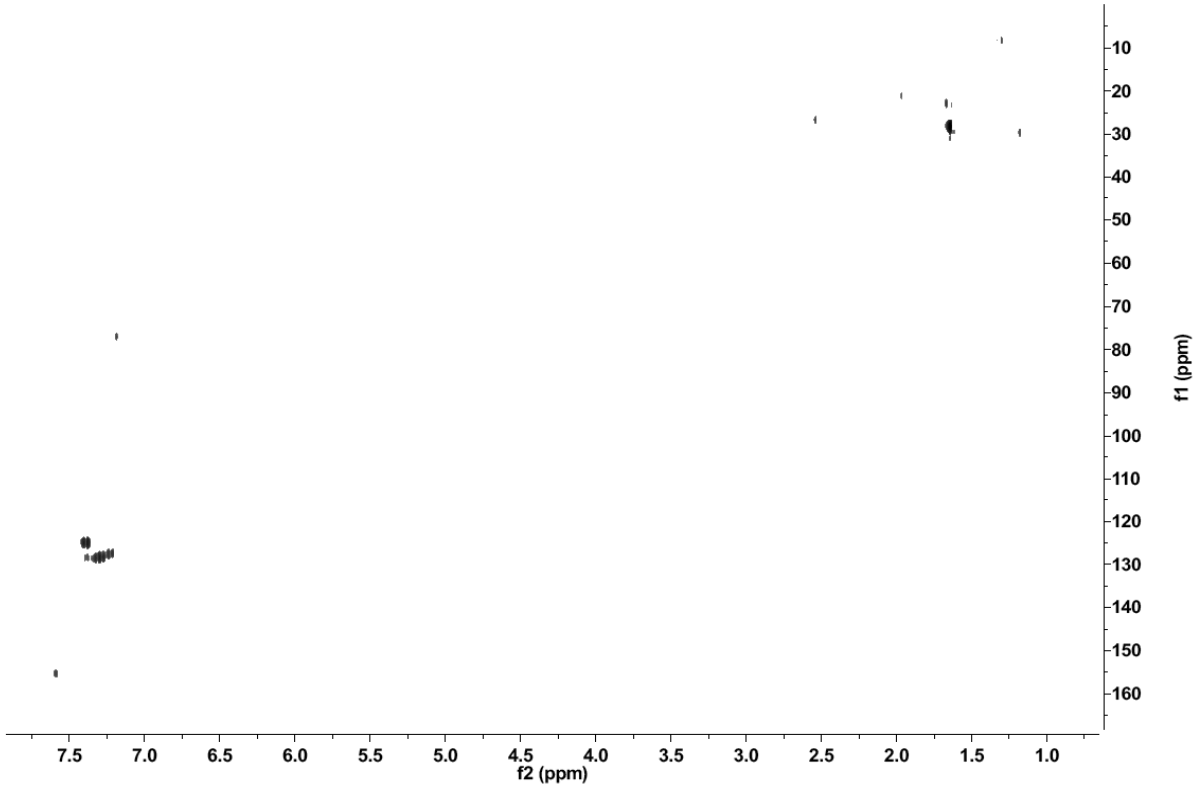


Figure S7 HSQC-NMR of oxazete **1e** (f1 = ^{13}C, f2 = ^{1}H)

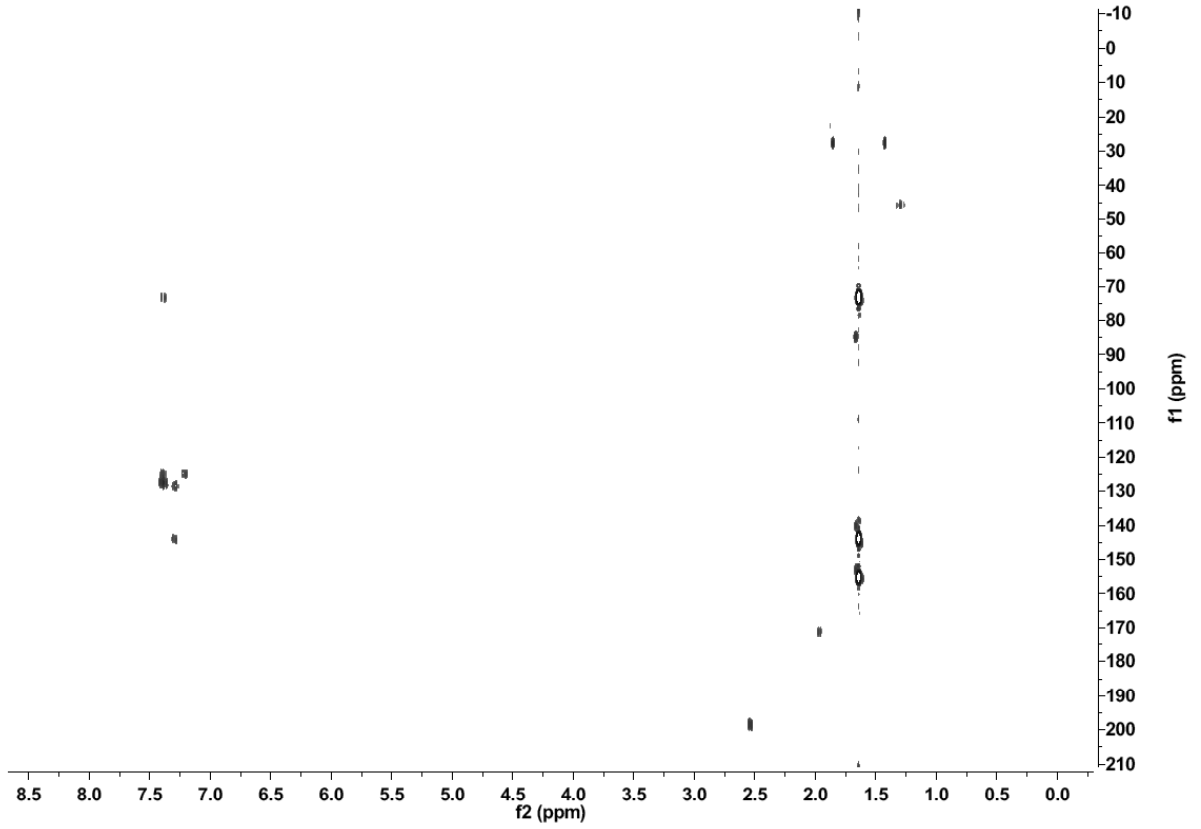


Figure S8 HMBC-NMR of oxazete **1e** (f1 = ^{13}C, f2 = ^{1}H)

Computational Details

Geometries were optimized using 2nd order Møller-Plesset perturbation theory[11] with the correlation consistent double-ζ basis set[12,13] (MP2/cc-pVDZ), followed by MP2/cc-pVTZ single point calculations. All structures were characterized as minima and transition states by MP2/cc-pVDZ frequency calculations. In addition, intrinsic reaction coordinate calculations[14] were used to verify that the located TSs connect the respective minima. Zero point energies and thermal correction to Gibbs free energies are unscaled. Higher-level correlation contributions were obtained by coupled-cluster[15] calculations [CCSD(T)/6-31G(d)]. Solvent effects (aqueous solution) were estimated by bulk solvation models, SM8[16,17] combined with the M06-2X[18] density functional [SM8-M06-2X/6-31+G(d,p)]. Programs used were NWChem 5.1.1,[19] GAMESS,[20] GAMESSPLUS,[21] Gaussian 03,[22] and MOLDEN[23] for visualization.

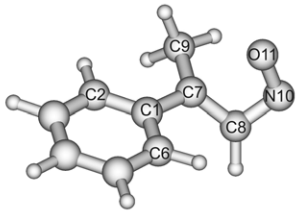


Figure S9 Calculated (MP2/cc-pVDZ) structure of the cyclization transition state (TS1)

Table S1 Calculated relative Gibbs free energies in gas phase and aqueous solution[a] and pertinent structural parameters (Figure 1).[b]

	ΔG(gas)	ΔG(H_2O)	τ_1	τ_2	τ_3	r(C7-O11)
(E)-trans-**1d**	-5.51	-5.63	179.1	-31.4 (147.9)	147.9 (-32.8)	3.474
TS1	3.68	5.61	93.1	-35.6 (143.9)	143.1 (-37.5)	3.134
(E)-cis-**1d**	0.00	0.00	26.4	-38.6 (141.5)	141.5 (-38.4)	2.823
TS2	29.24	22.20	21.9	-173.7 (4.4)	29.9 (-152.0)	2.098
Oxazete **1e**	-4.06	-8.40	-0.1	65.4 (-114.2)	-74.9 (105.6)	1.482
TS2’	31.97	26.10	21.7	33.6 (-141.7)	-167.3 (17.4)	2.086
(Z)-cis-**1d**	2.33	1.75	40.7	-58.9 (116.5)	-66.6 (118.0)	2.887
TS1’	4.68	5.70	95.7	-54.4 (123.9)	126.1 (-55.7)	3.173

(Z)-trans-**1d**	-3.96	-4.89	179.7	136.7 (-41.0)	139.3 (-43.0)	3.480

[a] ΔG (gas) = E(MP2/cc-pvTZ//MP2/cc-pVDZ) + {E[CCSD(T)/6-31G(d)] – E[MP2/6-31G(d)] + ΔG_{therm}; $\Delta G(H_2O)$ = ΔG (gas) + ΔG_{solv}[SM8-M06-2X/6-31+G(d,p)]. [b] τ_1 = τ(C7–C8–N10–O11); τ_2 = τ(C2 (C6)–C1–C7–C9); τ_3 = τ(C2 (C6)–C1–C7–C8). For atom numbering see Figure S4.

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