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PAPER

Reductive biotransformation of nitroalkenes *via* nitroso-intermediates to oxazetes catalyzed by xenobiotic reductase A (XenA)†

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A novel reductive biotransformation pathway for β,β-disubstituted nitroalkenes catalyzed by flavoproteins from the Old Yellow Enzyme (OYE) family was elucidated. It was shown to proceed *via* enzymatic reduction of the nitro-moiety to furnish the corresponding nitroso-alkene, which underwent spontaneous (non-enzymatic) electrocyclization to form highly strained 1,2-oxazete derivatives. At elevated temperatures the latter lost HCN *via* a retro-[2 + 2]-cycloaddition to form the corresponding ketones. This pathway was particularly dominant using xenobiotic reductase A, while pentaerythritol tetranitrate-reductase predominantly catalyzed the biodegradation *via* the Nef-pathway.

Introduction

The biodegradation of aromatic nitro-compounds predominantly proceeds through a three-step bioreduction of the nitro-group *via* the nitroso- and hydroxylamine-intermediates to furnish the corresponding aniline[1] or (especially for di- and tri-nitroarenes) *via* reduction of the electron-deficient aromatic system to form the so-called 'Meisenheimer-complex', which is stabilized *via* elimination of nitrite.[2] This metabolic activity is widespread among bacteria,[3] fungi, plants,[4] and yeasts.[5] The enzymes responsible for these reductive biotransformations are flavoproteins from the Old Yellow Enzyme (OYE) family.[6] In the context of these studies, xenobiotic reductases XenA and XenB from *Pseudomonas sp.*[7] and pentaerythritol tetranitrate (PETN) reductase from *Enterobacter cloacae*[8] have been aptly denoted as 'nitroreductases' for their ability to catalyze the reductive biodegradation of explosives such as PETN, trinitrotoluene (TNT) and nitroglycerin. It was recently reported that nitroreductase NRSal from *Salmonella typhimurium* constitutes the first single isolated enzyme catalyzing the reduction of nitrobenzene to aniline[9] accompanied by non-enzymatic (chemical) condensation of nitroso- and hydroxylamino-intermediates to yield azoxybenzene as a side product.[9,10] In contrast to the bioreduction of aromatic nitro-compounds, much less is known about nitroaliphatics. So far, the bioreduction of aliphatic nitro-compounds appears to be mainly associated with facultative anaerobic organisms,[11–13] which furnished the corresponding amines in low yields.[11,14] Aiming at the production of α-chiral primary amines in nonracemic form, we have recently investigated the bioreduction of aliphatic *sec*-nitro-compounds using oxygen-stable flavin-reductases from the OYE family. In contrast to our expectations, OYEs did not furnish amines, but reductively degraded aliphatic *sec*-nitro-compounds to the corresponding carbonyl-compounds *via* a cascade of enzymatic (nitro- and oxime)-reductions followed by spontaneous isomerisation/hydrolysis steps, which overall constitutes a biocatalytic equivalent to the Nef-reaction[15–18] (Scheme 1). During the Nef-pathway, the corresponding nitroso- and imine-intermediates could not be directly observed due to their inherent instability.[15,16] However, the corresponding oxime-intermediate could be unambiguously identified using (*E*/*Z*)-1-nitro-2-phenyl-1-propene (**1**) as substrate[15] (Scheme 2).

Results and discussion

Much to our surprise, the bioreduction of nitroalkene (*E*/*Z*)-**1** using xenobiotic reductase A (XenA) furnished the expected Nef-reaction products (nitroalkane **1a**, oxime **1b** and aldehyde **1c**) only in minor amounts (9% in total, Scheme 2, Table 1). In contrast, a new compound was detected by TLC and GC formed in 73% yield. The latter was sufficiently stable to allow chromatographic purification on silica gel. High resolution mass spectrometry (ESI) revealed a composition of $C_9H_{10}NO$ ($[M+H]^+$ *m*/*z* calcd. for 148.0762, found 148.0758). Analysis of DEPT-135, ^{1}H-, ^{13}C-NMR and two-dimensional HSQC and HMBC NMR spectra revealed oxazete **1e** as product. Multiplicities obtained from a DEPT-135 ($CDCl_3$) showed signals for δ 28.08 (CH_3), 73.46 (C_q), 125.06 (CH), 127.61 (CH), 128.54 (CH), 143.93 (C_q) and 155.24 (CH). Using 2D ^{1}H-^{13}C-HSQC and HMBC NMR spectra, the proton signals between 7.25 ppm and 7.51 ppm and the carbon signals at 125.1, 127.6, 128.5 and 143.9 ppm could be assigned to

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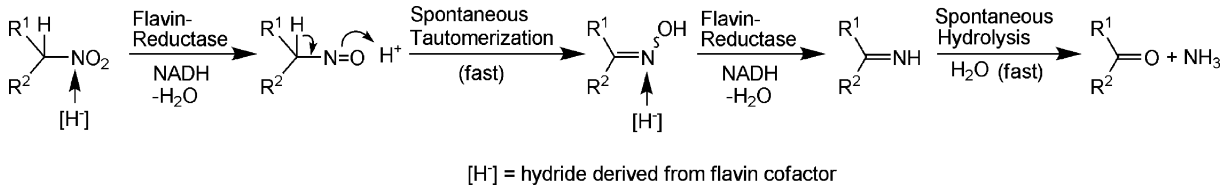
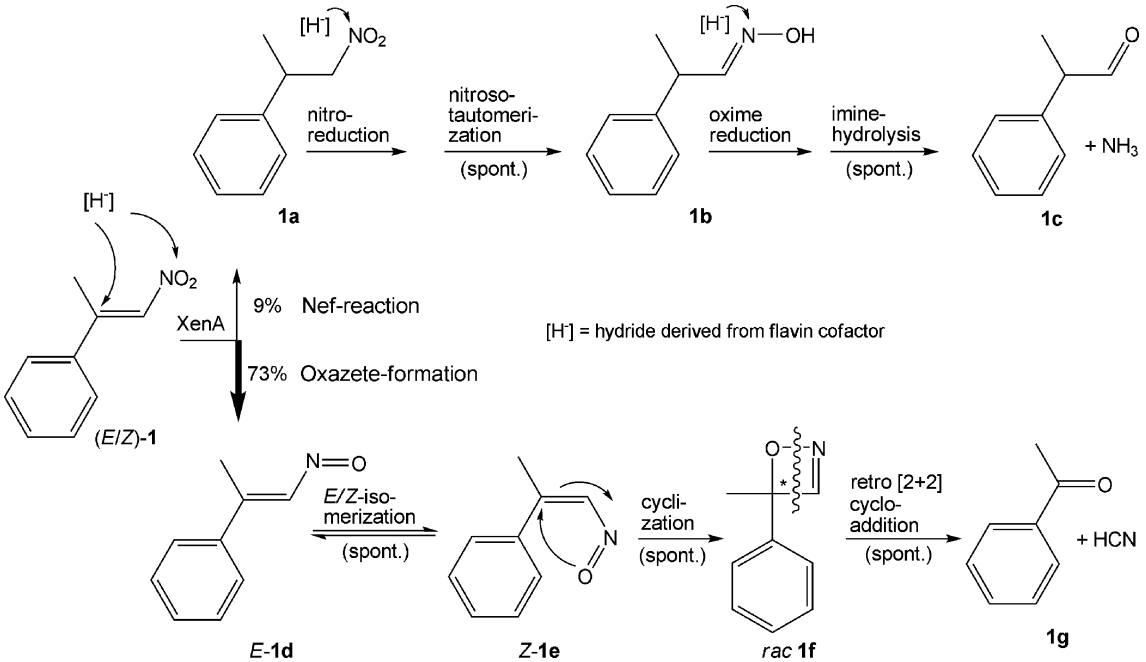


Scheme 1 Reductive biotransformation of nitroalkenes of OYEs *via* the Nef-pathway.



Scheme 2 Reductive biotransformation of nitroalkene (*E*/*Z*)-**1** using XenA.

Table 1 Bioreduction of (*E*/*Z*)-**1** using various OYEs in presence of NAD(P)H-recycling systems (Scheme 2)

			Conversion [%]				
Entry	Enzyme[a]	Conditions[b]	Nitroalkane **1a**	Oxime **1b**	Aldehyde **1c**	Oxazete **1e**	Acetophenone **1f**
1	XenA	NADH	2	5	2	51	22
2		NADPH	3	4	2	52	21
3		NAD^+/GDH/Glu	2	7	2	23	18
4		$NADP^+$/GDH/Glu	4	15	2	50	27
5	Mor-R	NADH	6	1	1	19	11
6		NAD^+/GDH/Glu	6	4	1	25	8
7	NerA	NADH	14	4	1	19	12
8	OPR3	NADH	24	4	2	15	16
9		NADPH	22	1	2	12	16
10		$NADP^+$/GDH/Glu	21	12	3	27	16
11	PETN	NADH	38	15	1	7	5
12		NADPH	49	21	1	8	6
13		NAD^+/GDH/Glu	49	26	2	9	3
14		$NADP^+$/GDH/Glu	48	32	2	12	4
15	XenB	NADH	66	8	1	7	8
16	YhdA	NADH	0	2	0	5	9

[a] XenA, XenB = xenobiotic reductase A and B, NerA = glycerol trinitrate reductase, MorR = morphinone reductase, OPR3 = 12-oxophytodienoic acid reductase, PETN-R = pentaerythritol tetranitrate reductase, YhdA = flavin-dependent quinone reductase. [b] Standard conditions: substrate (*E*/*Z*)-**1** (1 eq, 10 mM), Tris-buffer (50 mM, pH 7.5), NADH or NADPH (1.5 eq, 15 mM); cofactor recycling: NAD^+ or $NADP^+$ (100 μM), glucose dehydrogenase (10 U), glucose (2 eq, 20 mM); reaction time 24 h, shaking (120 rmp), 30 °C.

a mono-substituted phenyl group, with the quaternary carbon at 143.9 ppm being connected to the methyl protons at 1.74 ppm within three bonds according to the HMBC. This spectrum also showed correlations from the methyl protons to the carbon signals at 73.5 ppm and 155.2 ppm. A shift of 73.5 ppm for a quaternary carbon indicates a single bond to oxygen and the CH group at 155.2 ppm has to be a sp^2-hybrized carbon bound to a heteroatom, most likely nitrogen. Therefore, the methyl

group can only be bound directly to the quaternary carbon at 73.5 ppm, with the aromatic carbon at 143.9 ppm and the signal at 155.3 ppm being attached to the one at 73.5 ppm. Since no exchangeable proton was found and according to the sum formula, there is a single bond between oxygen and nitrogen leading to the proposed oxazete structure **1e**. Characteristic absorptions for C=N bonds (1555, 1602 and 1644 cm^{-1}) but no indications for C=O or nitro-groups were found in the IR.

In order to rationalize the unexpected formation of this highly strained heterocycle, the following pathway, which was supported by theoretical calculations[19–24] based on relative Gibbs free energies in the gas phase and in aqueous solution (SM8 bulk solvation model[25,26]) was elucidated (Fig. 1): in the first step, the nitro-moiety of (*E*/*Z*)-**1** was enzymatically reduced by a hydride delivered *via* the flavin cofactor to furnish the corresponding nitroso-alkene (*E*/*Z*)-**1d**.[27] The (*Z*)-isomer of the latter underwent electrocyclization yielding the highly strained four-membered heterocycle **1e**.[28]

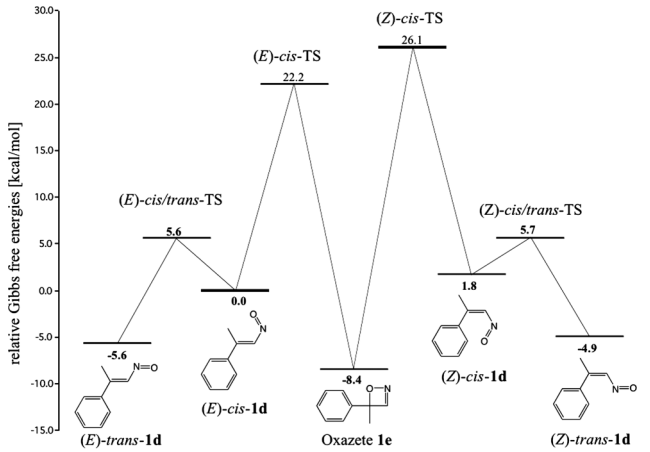


Fig. 1 Energy-diagram calculated for the cyclization of nitroso-alkene **1d** to oxazete **1e** in H_2O (TS = transition state).

An alternative mechanism for oxazete formation could be envisaged *via* conjugate addition of H_2O across the C=C bond of nitrosoalkene **1d**, thereby forming a benzylic *tert*-alcohol species which would undergo subsequent cyclisation with concomitant formation of water. Since the latter mechanism would involve nucleophilic attack of [OH^-] at the (sterically demanding) C_β of the nitrosoalkene, it was not considered as most plausible. 1,2-Oxazetes are known to occur as unstable intermediates in aza-Claisen rearrangements[29] and they have been postulated as intermediates during the formation of carbonyl-compounds and nitriles from vinyl-radicals and NO.[30] In addition, they are known to occur as reactive intermediates in thermal and photochemical reactions of α,β-unsaturated nitro-compounds.[31] Their stability can be significantly enhanced by sterically demanding substituents, which allow their synthesis *via* intramolecular cyclization of α,β-unsaturated nitroso-compounds[32] or by MCPBA-oxidation of oximes.[33]

According to our calculations, the (*E*)-configuration of nitroso-alkene **1d** is more stable than the (*Z*)-isomer, especially for the *s*-*cis*-conformation of the nitroso-group (Δ*G* (*E*/*Z*)-*cis* **1d** = 1.8 kcal mol^{-1}) (Fig. 1). Interconversion between the two rotamers, (*E*)-*trans*-**1d** → (*E*)-*cis*-**1d** and (*Z*)-*trans*-**1d** → (*Z*)-*cis*-**1d** occurs *via* a barrier of approximately 10 kcal mol^{-1}. In contrast to these rotational barriers, which are slightly larger in aqueous solution, the barrier for the cyclization is significantly lowered by solvent effects by *ca.* 6–7 kcal mol^{-1}. Moreover, the four-membered oxazete **1e** is preferentially stabilized by solvation, resulting in the greater stability of the product of *ca.* 2.5 kcal mol^{-1} compared to the most stable (*E*)-*trans*-nitrosoalkene **1d**. According to these calculations, the more unstable (*Z*)-*cis*-isomer of **1d** underwent cyclization to furnish **1e**.

Although oxazete **1e** proved to be reasonably stable at ambient temperatures, it underwent retro-[2 + 2]-cycloaddition at elevated temperatures forming acetophenone **1f** and HCN, which was proven by differential scanning calorimetry (DSC) measurements, which showed the highest HCN formation within a range of 120 °C to 160 °C, indicated by a broad sigmoidal shape of the DSC (Fig. S2, ESI†).

Since oxazete **1e** was racemic as demonstrated by GC using a chiral stationary phase and CD measurements (Fig. S1, ESI†), we concluded that the electrocyclization step is of a spontaneous nature and does not require the involvement of the flavoprotein.

In order to elucidate whether this oxazete formation is of a general nature or was an isolated phenomenon associated to XenA, nitroalkene **1** was tested with a broad range of OYEs using NADH or NADPH as cofactor, as well as in presence of the corresponding cofactor recycling systems. The data presented in Table 1 reveal that the type of pathway strongly depends on the nature of the enzyme:

(i) Using XenA, the oxazete-pathway was clearly dominant as indicated by the formation of oxazete **1e** and its degradation product acetophenone **1f** in up to 77% in total (entries 1–4). Morphinone reductase showed a similar, but somewhat less pronounced trend (entries 5 and 6, **1e** + **1f** up to 33%). With both enzymes, oxime/aldehyde formation yielding **1b** and **1c** (as an indicator of the Nef-pathway) constituted only a side-activity.

(ii) Glycerol trinitrate reductase NerA and 12-oxophytodienoic acid reductase OPR3 showed both remarkable oxazete formation (entries 7 and 10) going hand in hand with pronounced ene-reductase activity as indicated by the C=C-bond reduction of nitroalkene **1** forming nitroalkane **1a** in up to 21% (entry 10). Oxime formation (**1b**) was somewhat suppressed.

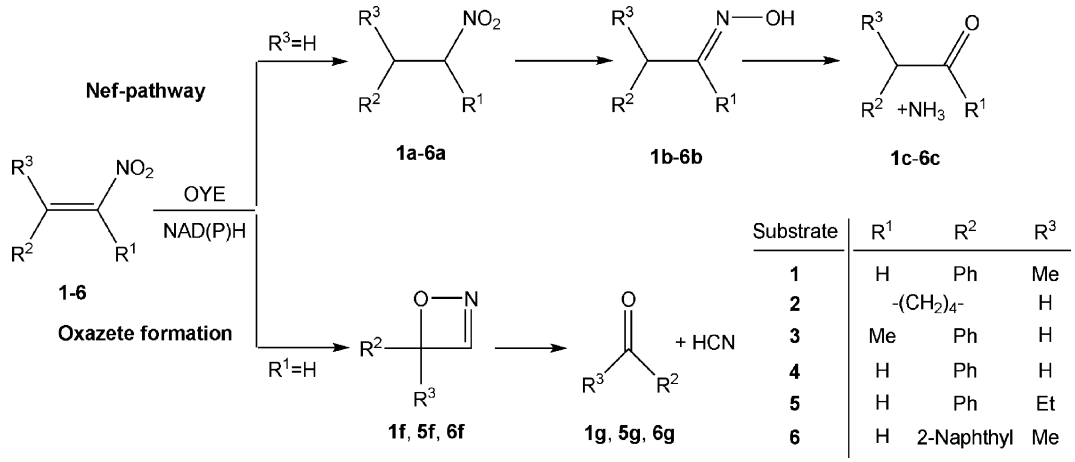
(iii) Pentaerythritol tetranitrate reductase showed only weak oxazete formation (**1e** up to 12%), but dominant alkene reduction (**1a** up to 49%) and remarkably high oxime formation (**1b** up to 32%, entry 14).

(iv) XenB predominantly proved to behave as an ene-reductase (**1a** 66%, entry 15), and the quinone reductase YhdA was almost inactive (entry 16).

Overall, there was no dramatic impact on the product distribution depending on the type of cofactor (NADH or NADPH) or on the respective cofactor recycling system employed. All blank experiments in the absence of protein and/or cofactor showed a conversion below the level of detectability (<3%).

In order to map the substrate tolerance for oxazete formation, a range of nitroalkenes **2–6** which are structurally related to substrate **1** was studied (Scheme 3).

The product distribution derived from the bioreduction of nitroalkenes **2–6** shows that oxazete formation is strongly dependent



Scheme 3 Bioreduction of substituted nitroalkenes **2–6** *via* the Nef-pathway *versus* oxazete formation.

on the substrate structure (Table 2). 1-Nitrocyclohexene (**2**) was mainly reduced at the C=C-bond yielding nitrocyclohexane (**2a**), the Nef-product cyclohexanone (**2c**) was formed in minor amounts (up to 12%) using OPR3. The corresponding oxazete pathway yielding **2e** and/or **2f** was not detectable. The open-chain substrate **3** gave similar results, *i.e.* mainly nitroalkane **3a** (XenB 96% **3a**) and Nef-degradation product **3c** (OPR3 15% **3c**) were formed. Both of the latter substrates share two α,β-substituents at the C=C-bond. In contrast, all enzymes solely gave the C=C-bond reduction product **4a** with the β-monosubstituted nitroalkene **4** in up to 67% conversion (XenB 67% **4a**). However, two β,β-substituents in substrate **5** opened the oxazete pathway, forming **5e** and its degradation product **5f**, this was most pronounced with XenA (27% **5e** + **5f**), while C=C-bond reduction remained low (OPR3 11% **5a**). Finally, a close analog of the original test substrate **1** bearing a 2-naphthyl group (**6**) was solely converted *via* the oxazete pathway, again XenA being best (41% **6e**+**6f**), no C=C-bond reduction was detected. Both oxazete derivatives **5e** + **5f**, **6e** + **6f** were characterised by GC-MS using compound **1e** as a lead. Overall, the corresponding oximes **2b–6b** were not detectable. From these results it can be concluded, that oxazete formation only takes place with β,β-disubstituted nitroalkenes (**1**, **5**, **6**) which – in line with the absence of an enantiomeric excess of **1e** – supports the hypothesis that it is of a non-enzymatic nature and proceeds *via* spontaneous electrocyclization of the corresponding nitrosoalkenes.

Table 2 Product distribution during bioreduction of nitroalkenes **2–6**

Substrate	Enzyme	Products[a] [%]									
2	XenA	**2a**	97	**2b**	<1	**2c**	3	**2e**	<1	**2f**	<1
	XenB		99				<1				
	NerA		90				9				
	OPR3		85				12				
3	XenA	**3a**	7	**3b**	<1	**3c**	<1	**3e**	<1	**3f**	<1
	XenB		96				3				
	NerA		88				11				
	OPR3		84				15				
4	XenA	**4a**	16	**4b**	<1	**4c**	<1	**4e**	<1	**4f**	<1
	XenB		67								
	NerA		<1								
	OPR3		13								
5	XenA	**5a**	5	**5b**	<1	**5c**	<1	**5e**	18	**5f**	9
	XenB		n.d.						n.d.		n.d.
	NerA		8						3		3
	OPR3		11						3		8
6	XenA	**6a**	<1	**6b**	<1	**6c**	<1	**6e**	13	**6f**	28
	XenB								<1		<1
	NerA								<1		15
	OPR3								<1		31

[a] Standard conditions: substrate **2–6** (1 eq, 10 mM), Tris-buffer (50 mM, pH 7.5), NADH (1.5 eq, 15 mM), reaction time 24 h, shaking (120 rmp), 30 °C; organic cosolvents were used to solubilize the following substrates: 20% v:v DMSO substrate **3**; *t*BuOMe (10% v:v) substrate **4**; *t*BuOMe (15% v:v) substrate **6**; XenA, XenB = xenobiotic reductase A and B, NerA = glycerol trinitrate reductase, OPR3 = 12-oxophytodienoic acid reductase; n.d. = not determined.

Conclusions

The reductive biotransformation of β,β-disubstituted nitroalkenes catalyzed by flavoproteins from the OYE family was shown to proceed not only *via* the recently elucidated Nef-reaction, but also *via* enzymatic reduction of the nitro-moiety instead of C═C-reduction to furnish the correponding nitroso-alkene, which spontaneously underwent electrocyclization to form highly strained 1,2-oxazete derivatives in racemic form. The latter were sufficiently stable to allow their structural characterization; at elevated temperatures they lost HCN *via* a retro-[2 + 2]-cycloaddition to form the corresponding ketones. The type of bioreduction pathway, *i.e.* Nef-pathway *versus* oxazete formation, depended not only on the substitutional pattern of the nitroalkene, but also on the type of flavoprotein: whereas PETN-reductase predominantly catalyzed the Nef-pathway, xenobiotic reductase A was strong in oxazete formation.

Experimental section

Cloning, expression and purification of XenA, XenB and NerA (GTN)

Xenobiotic reductase A from *Pseudomonas putida* (XenA), xenobiotic reductase B from *Pseudomonas putida* JLR11 (XenB), and glycerol trinitrate reductase from *Agrobacterium radiobacter* (NerA) were cloned, expressed and purified as follows. The DNA sequences were retrieved from the NCBI Genebank (accession files Q9R9V9 for XenA, Q9RPM1 for XenB and O31246 for NerA) and synthesized with a C-terminal hexahistidine tag. The genes were cloned into pET21a and transformed into the expression strain *E. coli* BL 21 (DE3). Heterologous expression of the genes in *E. coli* BL21 (DE3) was performed as follows: 100 ml starter LB_{Amp} cultures were inoculated with aliquots from a frozen stock culture and grown overnight at 37 °C. Each starter culture was used to inoculate a 700 ml culture, which was grown to an OD_{600} of 0.6–0.8. Gene expression was induced with 0.2 mM IPTG (final concentration) and protein expression was performed for 4 h at 37 °C. After centrifugation, cell pellets were frozen at −80 °C or used directly for the protein purification. The pellets were resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl and 20 mM imidazole) and sonicated on ice (t = 5 min, pulse 1 s, pause 2 s, amplitude 40%) with addition of FMN (*ca.* 3 mg). After centrifugation (30 min, 18 000 rpm, 4 °C), the supernatant was subjected to protein purification on a Ni^{2+}-NTA column according to standard protocol.[34]

General procedure for the bioreduction of substrates

An aliquot of enzyme [OPR3 isoenzyme of 12-oxophytodienoate reductase from *Lycopersicon esculentum*, PETN-reductase from *E. cloacae* PB2 (PETN-Red), morphinone reductase (Mor-Red), flavin-oxidase (YhdA), xenobiotic reductases (XenA, XenB) and glycerol trinitrate reductase (NerA), protein content ~100 μg ml^{-1}], was added to a Tris-HCl buffer solution (0.8 ml, 50 mM, pH 7.5) containing the substrate (10 mM) and the cofactor NADH or NADPH (15 mM). Alternatively, the oxidized form of the cofactor NAD^+ or $NADP^+$ (100 μM) was used in combination with a recycling enzyme (glucose dehydrogenase, 10 U) and the cosubstrate (glucose, 20 mM). The mixture was shaken at 30 °C and 120 rpm and after 24 h products were extracted with EtOAc (2 × 0.8 ml). The combined organic phases were dried (Na_2SO_4) and the resulting samples were analyzed on achiral GC or GC-MS after TLC control. For substrates of low solubility in Tris-HCl buffer cosolvents were used, **4**: 10% v:v *t*-butyl-methyl-ether (TBME); **3**: 20% v:v DMSO; **6**: 15% v:v TBME.

Synthesis and isolation of oxazetes

4-Methyl-4-phenyl-4*H*-1,2-oxazete (1e). The general procedure was up-scaled 60-fold using substrate (*E*/*Z*)-**1**. After the biotransformation all samples were pooled and extracted twice with 50 ml ethyl acetate. Removal of the solvent under reduced pressure gave an oily residue which was purified *via* flash chromatography on silica (ethyl acetate : petroleum ether 1 : 3) to yield 25 mg of pure 4-methyl-4-phenyl-4*H*-1,2-oxazete **1e**. TLC: R_f 0.30 (silica gel, ethyl acetate : petroleum ether 1 : 3, molybdate blue); GC-MS (EI): *m*/*z* 32, 43, 53, 63, 77, 91, 105, 122, 132, 148; HRMS (1.250.000 resolution, ESI) $(M + H)^+$ = *m*/*z* calcd. for $C_9H_{10}NO$ 148.0762, found 148.0758; IR (cm^{-1}): 1374, 1447, 1494, 1555, 1602, 1644, 2930, 2982. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.74 (3H, s), 7.25–7.35 (1H, t, J = 7.3 Hz), 7.35–7.44 (2H, t, J = 7.3 Hz), 7.46–7.51 (2H, d, J = 7.2 Hz), 7.68 (1H, br); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 28.1, 73.5, 125.1, 127.6, 128.5, 143.9, 155.2.

4-Ethyl-4-phenyl-4*H*-1,2-oxazete (5e). TLC: R_f 0.70 (silica, ethyl acetate : petroleum ether 1 : 3, molybdate blue); GC-MS (EI): *m*/*z* 32, 44, 51, 72, 77, 105, 134, 149, (161); **5f** *m*/*z* 32, 39, 44, 51, 55, 65, 77, 91, 104, 115, 119, 120. The data (GC, GC-MS, TLC) corresponded to **1e**.

4-Methyl-4-(naphthalen-2-yl)-4*H*-1,2-oxazete (6e). GC-MS (EI): *m*/*z* 32, 43, 57, 77, 127, 155, 170, 180 (197); **6f** *m*/*z* 32, 43, 56, 63, 77, 101, 127, 155, 170. The data (GC, GC-MS) corresponded to **1e**.

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