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Synthesis of γ-nitroaldehydes containing quaternary carbon in the α-position using a 4-oxalocrotonate tautomerase whole-cell biocatalyst†

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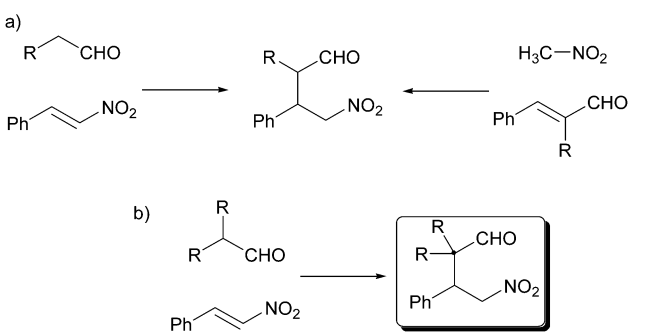
Synthesis of γ-nitroaldehydes from branched chain aldehydes and a range of α,β-unsaturated nitroalkenes was achieved by a whole-cell biocatalytic reaction using 4-oxalocrotonate tautomerase as catalyst. Under mild conditions, cyclic and acyclic branched aldehydes were converted into synthetically valuable quaternary carbon containing γ-nitroaldehydes. The yields of the desired products were influenced by reaction condition parameters such as organic solvent, temperature and pH. The whole-cell biocatalytic approach to the generation of α,α-substituted γ-nitroaldehydes was compared to the organocatalytic approach involving the lithium salt of phenylalanine as a catalyst. As the resulting γ-nitroaldehydes exhibited moderate antifungal activity and mild *in vitro* cytotoxicity against human fibroblasts (0.2–0.4 mM) they could further be examined as potentially useful pharmaceutical synthons.

Introduction

Synthetic methods allowing carbon–carbon bond formation under mild conditions are of great interest, especially when they address the problem of stereogenic quaternary carbon formation.[1,2] Quaternary carbon stereocenters (carbon centers containing four different non-hydrogen substituents) are present in a wide variety of organic substances, including bioactive natural products and medicinal agents.[3,4] Nevertheless, their generation still remains a challenge. Michael-type reactions are one of the most utilised reactions for the formation of C–C bonds in general, and they also afford the possibility of quaternary center introduction when α,α-disubstituted aldehydes are employed.[5–8] Furthermore, nitrostyrenes as highly reactive Michael acceptors are widely utilized in these type of reactions.[9] Michael-type addition is an effective method for obtaining synthetically useful γ-nitroaldehydes (Scheme 1). γ-Nitroaldehydes are precursors for γ-aminobutyric acid analogs (GABAs) exhibiting a range of pharmacological activities including antidepressant, anticonvulsant, anxiolytic and others.[10,11] There has been increasing interest in the synthesis of new GABA derivatives which can be potent drugs in the treatment of neurodegenerative disorders.[12] Furthermore, the presence of nitro group allows for further transformations to valuable functionalities such as amines.[13–15]

γ-Nitroaldehydes can be obtained by two different Michael-type additions: (i) from addition of aldehydes and α,β-unsaturated nitroalkenes and (ii) from addition of nitroalkanes and α,β-unsaturated aldehydes (Scheme 1a). However, in this



Scheme 1 Michael-type additions for introduction of quaternary carbon in the α-position.

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† Electronic supplementary information (ESI) available: Copies of the ^{1}H NMR and ^{13}C NMR spectra; supplementary Table 1. See DOI: 10.1039/c4ra05517a

system the only way to introduce quaternary centre in the α-position of nitroaldehyde is the reaction of branched aldehyde with a nitroalkene (Scheme 1b). Michael-type additions of unmodified aldehydes to nitroolefins were successfully developed through organocatalytic approaches.[16,17] They usually involve reactions of primary activated amines,[15,18–20] secondary amines,[19,21] alkaline metal salts of primary amino acids,[22,23] peptides[24] or functionalized chiral ionic liquids.[25] Although organocatalytic methods are versatile and valuable,[26] they are in this case dependant on application of excessive amounts of proline or peptide based catalysts (10–20% mol%)[24] and on the presence of usually chlorinated organic solvents, making their environmental impact high.

Only recently, due to the discovery of the versatile and promiscuous enzyme 4-oxalocrotonate tautomerase (4-OT) from *Pseudomonas putida* mt-2, which contains a terminal proline (Pro1) residue, Michael-type additions of aldehydes to nitroolefins were described.[27–29] It was proposed that the most likely catalytic mechanism of the 4-OT-catalyzed Michael-type additions also depend on proline-based catalysis and involves the formation of a nucleophilic enamine intermediate, which in turn reacts with the double bond of the nitro olefin creating a new C–C bond after which the product is released from 4-OT's Pro1 by hydrolysis.[27] Miao and co-authors have described that this particular enzyme in the purified form accepts in addition to acetaldehyde, a range of linear aldehydes up to octanal in the reaction with *trans*-nitrostyrene.[28] During the same study, they found that this system would not accept branched aldehydes including isobutanal nor acetone as donors.

Previously we have described a biocatalytic process intensification utilising whole-cell biocatalyst based on a 4-OT enzyme (*Escherichia coli* BL21 (4-OT)) originating from *Pseudomonas putida* mt-2 strain, in the biocatalytic synthesis of 4-nitro-3-arylbutanals.[30] Here we report on the extension of our biocatalytic strategy by showing that, 4-OT whole-cell biocatalyst also accepts variety of substituted aromatic and heterocyclic nitroalkenes as acceptors and branched aldehydes namely isobutanal, 2-ethylbutanal, cyclohexanecarbaldehyde and 2-methylpentanal as donors for the Michael-type addition (Scheme 2; Fig. 1A). In addition, we examined the antimicrobial and cytotoxic effects of resulting γ-nitroaldehydes (Fig. 1B). All products generated biocatalytically were also generated using asymmetric organocatalytic synthesis involving lithium salt of phenylalanine[22,23] allowing for the direct comparison of the two methods.

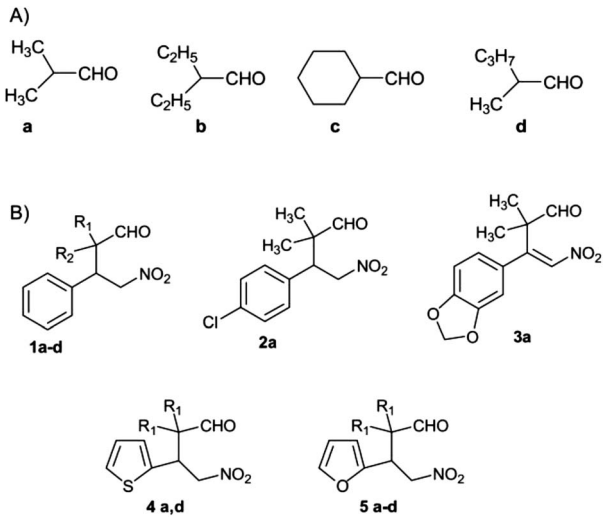


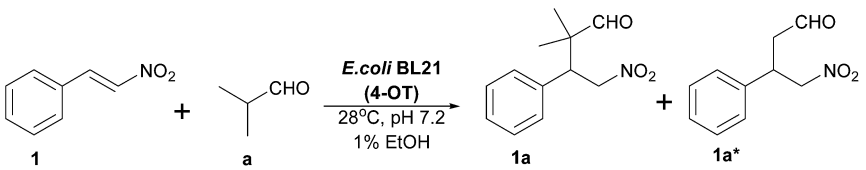
Fig. 1 Structures of (A) branched donors and (B) γ-nitroaldehyde products of Michael-type additions catalyzed by 4-OT.

Results and discussion

Biocatalytic synthesis of 2,2-dimethyl-4-nitro-3-phenylbutanal (1a) and influence of reaction conditions

We started our investigations by exploring the reaction of β-nitrostyrene (**1**) with isobutanal (**a**) using bacterial cells expressing 4-OT. A preparative scale reaction was performed with **1** (2 mM) and **a** (20 mM) in 20 mM NaH_2PO_4 buffer (pH 7.2) at 28 °C (Scheme 2). Due to the poor solubility of nitrostyrene in NaH_2PO_4 buffer, it was supplied from an ethanol stock solution (200 mM) so the reaction contained 1% (v/v) EtOH. As expected, upon completion (~4 h) 2,2-dimethyl-4-nitro-3-phenylbutanal (**1a**) was formed as a result of Michael-type addition of **1** and **a**. Surprisingly, significant amount of 4-nitro-3-phenylbutanal (**1a***), a product of Michael-type addition of acetaldehyde to **1**, was also isolated. The respective yields were 26 and 15% (Table 1, Entry I). In the control reaction with no microbial cells or autoclaved cells that should contain no enzymatic activity, no reaction occurred, while in the reaction of cells containing expression plasmid without the gene encoding the 4-OT enzyme, the reduction of **1** to 2-phenyl-nitroethane (5–10% yield) was observed regardless of the presence of aldehyde due to the presence of non-specific reductases in the host *E. coli* BL21 cells.[31] 2-Phenyl-nitroethane was not detected during biotransformations with *E. coli* BL21 (4-OT).

The addition of **1** (neat substance) to the aqueous reaction resulted in poor solubility and availability of the substrate,



Scheme 2 Tautomerase based whole-cell catalyzed Michael addition of isobutanal to β-nitrostyrene.

Table 1 Synthesis of **1a** (2,2-dimethyl-4-nitro-3-phenylbutanal) through biotransformation of β-nitrostyrene (**1**) and isobutanal (**a**) by recombinant *E. coli* BL21 (4-OT) using 5 g CDW L^{-1} at 28 °C, pH 7.2 in 60 mL volume

Entry	Solvent for **1**	Product	Reaction time (h)	**1** Depletion rate[a] (μmol per min per g CDW)	Yield (%)
I	Ethanol	**1a** and **1a***	4	1.8	26 and 15
II	Isopropanol	**1a**	4	1.7	54
III	2-Butanol	**1a**	4	1.7	60
IV	DMSO	**1a**	6	1.2	28
V	No	**1a**	20	0.21	10

[a] Standard errors were between 1 and 3%.

coinciding with a low substrate depletion rate and low yield of **1a** (10% after 20 h incubation) (Table 1; Entry V). Furthermore, no **1a*** was formed, suggesting that the presence of EtOH was crucial for the formation of **1a***. Thus we examined the influence of solvent used for the preparation of nitrostyrene stock solution on product formation and yield (Table 1). When isopropanol and 2-butanol were used for preparation of stock solution of **1**, overall yields of **1a** were 54 and 60%, respectively (Table 1; Entry II and III). When DMSO was used, product yield was approximately half of that obtained compared to when isopropanol and 2-butanol were used (Table 1; Entry IV). It is possible that an alcohol inducible dehydrogenase/acetaldehyde reductase was present in *E. coli* BL21 host cells and due to its activity, a portion of EtOH present in the reaction was converted to acetaldehyde that was further added to β-nitrostyrene by the activity of 4-OT resulting in formation of **1a*** (Table 1; Entry I). Indeed, numerous alcohol dehydrogenases including members of both short-chain and medium-chain dehydrogenase/reductases enzyme families have been described in *E. coli*.[32,33] In particular, ethanol-inducible ethanol dehydrogenase/acetaldehyde reductase, encoded by *adhP* or *yddN* gene was discovered relatively late due to inducibility of its activity by EtOH.[34] Recently, AdhP crystal structure was reported.[35] It was found that presence of 0.017 M EtOH induced AdhP activity, while the amount of EtOH in our reaction was 0.17 M.

The desired γ-nitroaldehyde **1a** was obtained in moderate enantioselectivity (48% ee; Table 3; Entry I). It was previously shown that specificity and control of stereochemical outcome of biocatalytic reactions could be achieved by adjustment of reaction conditions.[36,37] For example, pentaerythritol tetranitrate reductase enzyme scaffold was shown to be capable of generating both enantiomeric products with improved enantiopurities by a manipulation of the reaction conditions and/or in the presence of one or two key mutations.[36] Having in mind that the enzymatic activity of 4-OT mostly relies on Pro1 residue[29] and does not leave too much room for mutational improvements, we attempted to improve the stereoselectivity of Michael-type additions by lowering the pH of the reaction buffer to 5.5 and by reducing the reaction temperature to 20 °C and 10 °C. Overall, both types of adjustments had a negative effect on **1a** yield from the reaction catalyzed by whole-cell *Escherichia coli* BL21 (4-OT), while no improvement in ee values was observed (ESI Table 1†). This is in contrast to results obtained with purified 4-OT when lowering the pH from 7.0 to 5.5 in the addition reaction of acetaldehyde and β-nitrostyrene which caused an increase in product yield and reduced the amount of generated side products.[28] In the current study lowering the pH of reaction buffer from 7.2 to 5.5 resulted in a considerably lower product yield (26% instead 60%). The observed reduction of the product yield to 26% and 15% (ESI Table 1;† Entry II and IV) could be explained by the poorer transfer rates across the membrane of the substrates under these reaction conditions. Control reaction using cell-free extracts of the *E. coli* BL21 (4-OT) as catalyst required longer time for the Michael adduct to be formed, but decrease of the product yield was not observed (ESI Table 1;† Entry II′–IV′). This may be due to the fact that lowering the pH of the buffer in the case of whole-cells did not change the pH inside the cells, where the enzyme is located, while it negatively affected substrate uptake or transport. In the case of both purified enzyme[28] and whole-cell biocatalyst, lowering the pH of the reaction buffer did not affect the enantioselectivity (ESI Table 1;† Entry II).

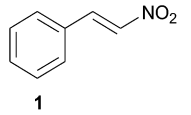
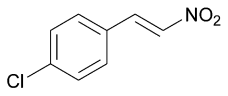
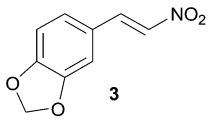
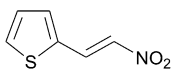
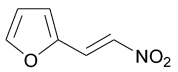
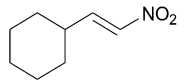
As no improvement of stereoselectivity was observed when reaction conditions were adjusted or different co-solvents were used while the reaction rates were comparable, all subsequent reactions were carried out in the presence of 2-butanol as reactions with it resulted in highest product yield.

Biotransformation of a range of α,α-substituted aromatic and heterocyclic nitroalkenes

With the best conditions of reaction defined, we explored if substrate scope could be expanded to substituted aromatic (**2** and **3**), heterocyclic (**4** and **5**) and nonaromatic (**6**) nitroalkenes in the reaction with isobutanal (**a**) (Table 2).

p-Chloro-*trans*-β-nitrostyrene (**2**) and 3,4-(methylenedioxy)-β-nitrostyrene (**3**), as well as (*E*)-2-(thiophen-2-yl)nitroethene (**4**) and (*E*)-2-(furan-2-yl)nitroethene (**5**) were biotransformed into corresponding nitroaldehydes, while the only substrate that could not be utilized as acceptor in this reaction was (*E*)-2-cyclohexyl-1-nitroethene (**6**) (Table 2; Entry VI). This was not surprising due to significant difference between benzene and cyclohexane ring in their steric and electronic properties. Hence, the aromatic nature of the ring was concluded necessary for reaction to occur. While reaction with **1** was completed within 4 h, longer reaction times (24 h) were required for heteroaromatic Michael adducts **4a** and **5a** (thiophen-2-yl and furan-2-yl) to be formed (Table 2; Entry IV and V). Desired product yields, as estimated from the GC analysis were approximately 2-fold lower for **4a** and **5a** in comparison to **1a**

Table 2 Synthesis of γ-nitroaldehydes through biotransformation of different nitroalkenes (**1–6**) and isobutanal (**a**) by recombinant *E. coli* BL21 (4-OT) using 5 g CDW L^{-1} at 28 °C, pH 7.2 in 60 mL volume

Entry	Substrate[a]	Product	Reaction time (h)	Yield[b] (%)
I	 **1**	**1a**	4	71
II	 **2**	**2a**	48	18
III	 **3**	**3a**	48	12
IV	 **4**	**4a**	24	37
V	 **5**	**5a**	24	39
VI	 **6**	nr[c]	—	—

[a] Nitroalkenes (**1–6**) were added to reaction as stock solution in 2-butanol. [b] Estimated yield based on GC-MS analysis. [c] No reaction occurred after 72 h incubation.

with by-product of substrate reduction occurring at much higher percentage between 30–40% (results not shown). On the other side substituted aromatic acceptors **2** and **3** when utilized in the reaction with isobutanal (**a**) even after prolonged incubation of 48 h afforded only 18 and 12% of desired γ-nitroaldehydes products, respectively. The biotransformation product mixture of these two substrates was very complex containing high amounts untransformed starting material (40–50%) and reduction and other degradation by-products (20–30%). Overall, **2a** and **3a** were the least represented in the corresponding biotransformations mixtures, hampering their successful isolation and purification, so **2** and **3** were not included as substrates in the subsequent study. This was surprising, as in the similar reaction using the same biocatalyst and acetaldehyde as donor, *p*-chloro-*trans*-β-nitrostyrene (**2**) was successfully transformed to 3-(4-chlorophenyl)-4-nitrobutanal with 38% isolated product yield,[30] suggesting the steric importance of the Michael donor in this reaction.

Different branched aldehydes as donors in Michael-additions catalyzed by 4-OT

It was explored if 2-ethylbutanal (**b**), cyclohexanecarbaldehyde (**c**) and 2-methylpentanal (**d**) could be employed as donors in Michael-type additions catalyzed by 4-OT whole-cell biocatalyst (Fig. 1). Based on the results of the initial reaction with isobutanal, three different Michael acceptors **1**, **4** and **5** were included in this part of the study.

When β-nitrostyrene was used as substrate, corresponding γ-nitroaldehydes **1b–d** bearing an all-carbon quaternary center in the α-position were obtained in good yields (Table 3; Entry II–IV). It is worth mentioning that although depletion of the conversions of substrates were 100%, isolated products yields were usually lower in comparison to the yield estimated by GC analysis, suggesting that percentage of the product was not successfully recovered during the purification procedure. Reactions with sterically demanding α,α-dialkyl aldehydes (**b–d**) with β-nitrostyrene required longer reaction time and afforded products with lower enantioselectivity (Table 3; Entry II–IV). This was also the case when **4** and **5** were used as Michael acceptors (Table 3; Entry VI and VIII–X). Unfortunately, corresponding Michael adducts when (*E*)-2-(thiophen-2-yl) nitroethene (**4**) was used as acceptor were not obtained in reactions with **b** and **c**. The lower enantioselectivity in comparison to the reaction when isobutanal (**a**) was used as substrate may be due to the structural extension causing steric effect and substrate conformation of aldehydes **b–d** or even retro-Michael reaction followed by side processes. The trend that substrate conformation governs enantioselectivity of the process was previously observed in Michael additions of unmodified aldehydes with nitroolefins catalyzed by pyrrolidine sulfonamide.[15] Indeed, similar observation was reported by Yoshida and co-authors when various α-branched and unbranched aldehydes were tested as Michael donors in reaction with β-nitrostyrene catalyzed by phenylalanine lithium salt, when bulkier aldehydes required longer reaction times and asymmetric α-branched aldehydes such as 2-phenylpropionaldehyde caused reduced enantioselectivity.[23] It appeared that L-phenylalanine lithium salt as catalyst afforded γ-nitroaldehydes in good yields with much higher enantioselectivity in comparison to our biocatalytic approach using whole-cells expressing 4-oxalocrotonate tautomerase, therefore we have applied the reported organocatalytic method in the synthesis of **1a–d**, **4a**, **4d** and **5a–d** for direct comparison (Table 3: Entries I′–X′).

In direct comparison isolated product yields were generally higher in synthesis using asymmetric organocatalytic approach (Table 3; gray shaded rows) with ee values between 92 and 96%, with reaction times comparable or longer and the reaction medium was CH_2Cl_2. Interestingly, when 2-ethylbutanal (**b**) was used as Michael donor, biocatalytic approach was superior in terms of product yield and the reaction time (Table 3; Entry II and II′). In the case when non-symmetrically branched aldehyde **d**, slightly better diastereoselectivity of Michael adducts was observed by NMR analysis in organocatalytic approach (Table 3; Entry IV′, VI′ and X′). Great differences in two approaches, especially in terms of enantioselectivity could be explained by the fact that the lithium cation behaves as a Lewis acid to aid the formation of enamine between the catalyst and aldehydes and enables favourable transition state.[23,38] However, a secondary amino acid L-proline and its lithium salt were not

Table 3 Comparative synthesis of γ-nitroaldehydes through transformation of α,β-substituted nitroalkenes and branched aldehydes by recombinant tautomerase and using lithium salt of phenylalanine

Entry	Substrate[a]	Product	Reaction time (h)	Isolated yield (%)	dr[b]	ee[c] (%)
I	**1**	**1a**	4	60	—	48
I′	**1**	**1a**	24	52	—	94
II	**1**	**1b**	16	70	—	9
II′	**1**	**1b**	72	31	—	92
III	**1**	**1c**	20	57	—	17
III′	**1**	**1c**	24	41	—	95
IV	**1**	**1d**	20	59	64/36	—[d]
IV′	**1**	**1d**	24	58	74/26	—
V	**4**	**4a**	24	34	—	10
V′	**4**	**4a**	24	78	—	94
VI	**4**	**4d**	24	32	67/33	—
VI′	**4**	**4d**	24	70	75/25	—
VII	**5**	**5a**	24	37	—	2
VII′	**5**	**5a**	24	60	—	96
VIII	**5**	**5b**	36	10	—	8
VIII′	**5**	**5b**	72	12	—	94
IX	**5**	**5c**	36	35	—	6
IX′	**5**	**5c**	24	69	—	94
X	**5**	**5d**	36	27	64/36	—
X′	**5**	**5d**	24	67	74/26	—

[a] Substrates were added to reaction as stock solution in 2-butanol. [b] Determined by ^{1}H NMR. [c] Determined by chiral HPLC analysis using Chiralpak IA. [d] Not determined because of insufficient chromatographic separation of enantiomers.

able to catalyse this Michael-type addition, as reported in the same study.[23] As mentioned, catalytic mechanism of 4-OT in Michael addition relies on promiscuous activity of this enzyme and terminal proline residue that participates in the formation of a nucleophilic enamine intermediate.[27]

Other organocatalytic approaches were also employed in synthesis of some of the γ-nitroaldehydes obtained in this study. 1-(2-Nitro-1-phenylethyl)cyclohexanecarbaldehyde (**1c**) was previously obtained by organocatalysis.[15,39–42] In most cases product yields reported were between 31–51% which is comparable to our biocatalytic result but reaction times were about 3–5 times longer (70–96 h *vs.* 20 h). The best organocatalytic approach to produce **1c** with a reported yield of 90% and a reaction time of 24 h was performed using rather specialised chiral amine/acid bifunctional catalyst (0.3 equiv.).[18]

This is the first time that 2,2-diethyl-4-nitro-3-phenylbutanal (**1b**), 2-methyl-2-(2-nitro-1-(thiophen-2-yl)ethyl)pentanal (**4d**), 2,2-diethyl-3-(furan-2-yl)-4-nitrobutanal (**5b**) and 2-(1-(furan-2-yl)-2-nitroethyl)-2-methylpentanal (**5d**) were synthesized and characterized using biocatalytic and organocatalytic approach.

Antimicrobial properties and cytotoxicity of γ-nitroaldehydes

Pharmacological effects of GABA and analogs are usually generated by interaction with both $GABA_{(B)}$ and gamma-hydroxybutyric acid (GHB) receptors, as well as from the influence on other transmitter systems in the human brain.[11] On the other hand, β-nitrostyrene derivatives have shown potential antibacterial activities.[43] To the best of our knowledge, γ-nitroaldehydes have not been examined previously for antimicrobial activity and their cytotoxic properties have not been reported.

As γ-nitroaldehydes were obtained in sufficient amounts they were screened against a range of Gram-positive and Gram-negative bacteria and exhibited no significant antibacterial activity including *Micrococcus luteus*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Klebsiella pneumoniae* (results not shown). However, antifungal activity against *Candida albicans* was more prominent (Table 4).

Due to structural similarity the activity of generated aldehydes was compared to that of β-nitrostyrene as a known antifungal agent.[44] *C. albicans* MIC values of γ-nitroaldehydes were higher in comparison to the MIC value of **1**, but they also exhibited much less cytotoxicity against human fibroblasts *in vitro* (Table 3). Nitrostyrene has also previously been found to be cytotoxic on stomach cancer lines in doses of 0.75 μg mL^{-1} and to have adverse effects on the immune system.[45] Of particular interest was 2,2-diethyl-4-nitro-3-phenylbutanal (**1b**) with good antifungal activity and cytotoxic effect exhibited in 2.5- and fold higher concentrations than MIC values against fungal strain (Table 4). Still, MIC values against *C. albicans* obtained for γ-nitroaldehydes are higher than those of antifungal agents clinically available such as nystatin which MIC against the same strain of *C. albicans* was determined to be 5–10 μg mL^{-1}.[46] However, nystatin and other clinically used antifungals also exhibit potent cytotoxicity; therefore search for novel antifungal agents is ongoing effort.[47]

Table 4 *In vitro* antifungal and anti proliferative activity of nitrostyrene and γ-nitroaldehydes generated in this study against *Candida albicans* and MRC5 fibroblast cell line

	MIC[a] (μg mL^{-1})	IC_{50}[b] (μg mL^{-1})
Compound	*Candida albicans* ATCC 10259	MRC5
1	10 ± 1	0.5 ± 0.1
1a	100 ± 3	50 ± 1
1b	40 ± 1	100 ± 2
1c	150 ± 4	60 ± 1
1d	60 ± 5	50 ± 2
4a	170 ± 7	60 ± 2
4d	40 ± 2	60 ± 2
5a	50 ± 4	40 ± 1
5b	100 ± 5	40 ± 2
5c	80 ± 3	80 ± 2
5d	200 ± 6	30 ± 1

[a] MIC values (minimal inhibitory concentrations) are calculated from three independent experiments and are expressed as means ± SD. [b] IC_{50} values (concentrations at which 50% cell growth inhibition occurs) are calculated from three independent experiments and are expressed as means ± SD.

Conclusion

Previously reactions of 4-oxalocrotonate tautomerase whole cell biocatalyst with acetaldehyde and a range of β-nitrostyrenes were rapid and yielded products with high enantiopurity.[30] During this study the scope of the biocatalytic reaction based on 4-OT was significantly widened, so that biotransformations with both acyclic and cyclic branched aldehydes afforded good route to γ-nitroaldehydes containing quaternary carbon in α-position but showed reduced enantiopurity. Although biocatalytic route offers mild reaction conditions and thereby safer and environmentally friendlier synthesis, we have also shown that asymmetric organocatalytic approach in synthesis of these products is superior in terms of enantiopurity. Regardless of the applied approach, γ-nitroaldehydes with an all carbon quaternary center in α-position provide an easy production route to novel γ-amino acids, γ-butyrolactones and substituted pyrrolidines. In addition to their structural similarity to GABA precursors, newly synthesised γ-nitroaldehydes were shown to have other biological activities such as antifungal.

Experimental

Materials

β-Nitrostyrene (**1**), 3,4-(methylenedioxy)-β-nitrostyrene (**3**), (*E*)-2-(thiophen-2-yl)nitroethene (**4**), isobutanal, 2-ethylbutanal, cyclohexane-carbaldehyde and 2-methylpentanal were obtained from Sigma Aldrich (Munich, Germany). Nitroalkenes **2**,[48] **5**[49] and **6**[50] were prepared according to the published procedures. Ethylacetate, ethanol and other solvents were of HPLC reagent grade and purchased from Fisher Scientific (Hampton, NH, USA). All solvents were freshly distilled before use.

Bacterial media components, casamino acids, ampicillin, isopropyl-β-D-thiogalactopyranoside (IPTG) were purchased from Sigma-Aldrich (Munich, Germany) or Fisher Scientific (Hampton, NH, USA) unless otherwise stated, and used without further purification.

Biocatalyst preparation and representative biocatalytic reaction

Whole-cell biocatalyst (*Escherichia coli* BL21 (4-OT)) used in this study was generated in our laboratory and recently described.[30] Briefly, this strain is recombinantly expressing 4-oxalocrotonate tautomerase (4-OT) from *Pseudomonas putida* mt-2 with inducible expression under control of T7 polymerase. For the biotransformations, cultures were grown in M9 medium[51] supplemented with glucose (1%, w/v), casamino acids (0.5%, w/v) and ampicillin (50 μg mL^{-1}) at 30 °C with shaking at 200 rpm. Once the culture had reached an optical density of 0.5 (600 nm; Spectophotometer Ultrospec 3300pro, Amersham Biosciences), cells were induced with 0.1 mM IPTG at 28 °C. After 12 h incubation, wet cell pellets were resuspended to a concentration of cell dry weight (CDW) of 5 g L^{-1} ($OD_{600} = 20$) in 20 mM phosphate buffer pH 7.2. Cell free extracts of the *E. coli* BL21 (4-OT) cells were prepared in a similar way, by disrupting cell membrane using Ultrasonicator (5 cycles of 10 s pulse and 20 s pause) and removing cell debris using centrifugation.

The whole cell biotransformation was generally carried out in 250 mL Duran Schott glass bottles with screw type plastic lids containing 60 mL cells suspension at 28 °C with shaking at 150 rpm. Unless otherwise stated, α,β-unsaturated nitroalkenes (**1–6**) were added sequentially as described previously[30] to a final concentration of 2 mM (18 mg, 0.12 mmol) from a 200 mM stock solution in 2-butanol and aldehydes **a–d** (1.2 mmol) were added to 20 mM final concentration. Samples (800 μL) were withdrawn from the reaction over time, centrifuged at 13 000 × *g* for 5 min and supernatants analyzed spectrophotometrically as previously described, following the depletion of **1** by reduction of absorbance at 320 nm ($\varepsilon = 14.4$ mM^{-1} cm^{-1}).[29]

To extract and purify the products from the biotransformation, the reaction mixture was extracted with ethylacetate (2 × 100 mL). The combined organic extract was washed with brine (100 mL), dried over anhydrous $MgSO_4$. After filtration and removal of the solvent under reduced pressure, the residue was purified by dry flash column chromatography (silica gel), eluting with petroleum ether–ethylacetate mixture in gradient (from 7 : 3 to 9 : 1) to afford pure nitroaldehydes.

General procedure for the synthesis of γ-nitroaldehydes from the corresponding aldehydes catalysed by L-phenylalanine lithium salt was carried out using reported procedures.[22,23]

Analytical methods (HPLC)

The enantiomeric excess was determined by HPLC (Agilent Technologies, HP110) with Chiralpak IA column (Chiral Technologies Europe, Cedex, France) at 210 nm for all samples. Racemic mixture of the samples was prepared chemically from the corresponding aldehydes using reported procedure.[48]

NMR spectra were recorded on a Varian Gemini 200 (^{1}H NMR at 200 MHz, ^{13}C NMR at 50 MHz, for samples in deuterated chloroform), and on Bruker Avance III 500 (^{1}H NMR at 500 MHz, ^{13}C NMR at 125 MHz). Chemical shifts are expressed in ppm (δ) using tetramethylsilane as internal standard, coupling constants (J) are in Hz. IR spectra were recorded on a Nicolet 6700 FT instrument, and are expressed in cm^{-1}. Mass spectra were obtained on Agilent technologies 6210 TOF LC/MS instrument (LC: series 1200). GC/MS analyses were performed on Agilent technologies 7890A-5975C inert XL EI CI instrument.

Characterization of γ-nitroaldehydes

2,2-Dimethyl-4-nitrophenylbutanal (**1a**)[23] ^{1}H NMR (200 MHz, $CDCl_3$) δ 9.53 (s, 1H), 7.40–7.13 (m, 5H), 4.92–4.64 (m, 2H), 3.78 (dd, $J_1 = 11$ Hz, $J_2 = 4.4$ Hz, 1H), 1.14 (s, 3H), 1.01 (s, 3H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 204.3, 135.3, 129.0, 128.7, 128.1, 76.3, 48.3, 48.2, 21.6, 18.8; HPLC (Chiralpak IA, i-propanol–heptane = 20/80, flow rate 1 mL min^{-1}, $\lambda = 210$ nm): $t_{major} = 2.98$ min, $t_{minor} = 4.01$ min; IR ν_{max}: 2975, 2935, 1725, 1554.

2,2-Diethyl-4-nitro-3-phenylbutanal (**1b**) ^{1}H NMR (200 MHz, $CDCl_3$) δ 9.58 (s, 1H), 7.38–7.11 (m, 5H), 4.85–4.58 (m, 2H), 3.33 (t, $J = 7.4$ Hz, 1H), 1.80–0.86 (m, 10H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 207.8, 135.2, 129.1, 128.9, 128.8, 128.5, 128.1, 127.4, 48.1, 33.4, 23.5, 22.6, 7.9, 7.5. IR ν_{max}: 2972, 2941, 1718, 1553. HRMS (ESI): m/z calcd for $C_{14}H_{23}N_2O_3$: 267.1703 $[M + NH_4]^+$; found 267.1693. HPLC (Chiralpak IA, i-propanol–heptane = 30/70, flow rate 1 mL min^{-1}, $\lambda = 210$ nm): $t_{major} = 3.87$ min, $t_{minor} = 4.72$ min.

1-(2-Nitro-1-phenylethyl)cyclohexanecarbaldehyde (**1c**)[18] ^{1}H NMR (200 MHz, $CDCl_3$) δ 9.56 (s, 1H), 7.63–7.02 (m, 5H), 4.90–4.58 (m, 2H), 3.54 (dd, $J_1 = 10$ Hz, $J_2 = 5.6$ Hz, 1H), 2.39–0.85 (m, 10H), ^{13}C NMR (50 MHz, $CDCl_3$). δ 207.3, 134.8, 129.1, 128.7, 128.1, 76.3, 51.2, 50.4, 30.9, 29.6, 25.0, 22.6, 22.5; IR ν_{max}: 2933, 2856, 1719, 1553. HPLC (Chiralpak IA, i-propanol–heptane = 30/70, flow rate 1 mL min^{-1}, $\lambda = 210$ nm): $t_{major} = 4.13$ min, $t_{minor} = 4.89$ min.

2-Methyl-2-(2-nitro-1-phenylethyl)pentanal (**1d**)[18] ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.54 (major isomer) and 9.52 (minor isomer) (s, 1H), 7.34–7.27 (m, 3H), 7.21–7.16 (m, 2H), 4.85 (minor isomer) and 4.84 (major isomer) (dd, $J = 11.3$, 13.1 and 11.5, 13.0 Hz, 1H), 4.76 (minor isomer) and 4.63 (major isomer) (dd, $J = 4.3$, 13.1 and 3.9, 13.0 Hz, 1H), 3.79 (major isomer) and 3.77 (minor isomer) (dd, $J = 3.9$, 11.4 and 4.3, 11.2 Hz, 1H), 1.63–1.17 (m, 4H), 1.11 (major isomer) and 1.10 (minor isomer) (s, 3H), 0.9 (minor isomer) and 0.84 (major isomer) (t, $J = 7.2$ and 6.9 Hz, 3H).

2,2-Dimethyl-4-nitro-3-(thiophen-2-yl)butanal (**4a**)[23] ^{1}H NMR (200 MHz, $CDCl_3$) δ 9.54 (s, 1H), 7.27–7.22 (m, 1H), 7.00–6.91 (m, 2H), 4.75–4.59 (m, 2H), 4.14 (dd, $J_1 = 4.9$ Hz, $J_2 = 10.0$ Hz, 1H), 1.21 (s, 3H), 1.09 (s, 3H); HPLC (Chiralpak IA, i-propanol–heptane = 10/90, flow rate 1 mL min^{-1}, $\lambda = 210$ nm): $t_{minor} = 8.08$ min, $t_{major} = 7.82$ min.

2-Methyl-2-(2-nitro-1-(thiophen-2-yl)ethyl)pentanal (**4d**) ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.54 (s, 1H), 7.26–7.22 (m, 1H), 6.98–6.89 (m, 2H), 4.78–4.60 (m, 2H), 4.20 (major isomer) and 4.12 (minor isomer) (dd, $J = 3.8$, 11.1 and 4.5, 10.4 Hz, 1H), 1.69–1.22 (m, 4H), 1.20 (major isomer) and 1.17 (minor isomer) (s, 3H), 0.92 (minor isomer) and 0.85 (major isomer) (t, $J = 7.2$ and 7.1 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 204.6 (major isomer) and 204.2 (minor isomer), 137.8, 127.93 (major isomer) and 127.86 (minor isomer), 126.9, 125.58 (minor isomer) and 125.46 (major isomer), 78.2, 51.8 (major isomer) and 51.1 (minor isomer), 44.4 (minor isomer) and 42.4 (major isomer), 37.6 (major isomer) and 36.4 (minor isomer), 17.1 (minor isomer) and 17.0 (major isomer), 16.0, 14.5 (minor isomer) and 14.3 (major isomer). IR ν_{max}: 2963, 2935, 1723, 1556. HRMS (ESI): m/z calcd for $C_{12}H_{21}N_2O_3S$: 273.1267 $[M + NH_4]^+$; found 273.1270.

3-(Furan-2-yl)-2,2-dimethyl-4-nitrobutanal (**5a**)[23] ^{1}H NMR (200 MHz, $CDCl_3$) δ 9.53 (s, 1H), 7.38 (d, $J = 1.6$ Hz, 1H), 6.32 (dd, $J_1 = 1.6$ Hz, $J_2 = 3.2$ Hz, 1H), 6.20 (d, $J = 3.2$ Hz, 1H), 4.76 (dd, $J_1 = 10.9$ Hz, $J_2 = 12.9$ Hz, 1H), 4.59 (dd, $J_1 = 4.1$ Hz, $J_2 = 12.9$ Hz, 1H), 3.92 (dd, $J_1 = 4.1$ Hz, $J_2 = 10.9$ Hz, 1H), 1.18 (s, 3H), 1.05 (s, 3H); HPLC (Chiralpak IA, i-propanol–heptane = 10/90, flow rate 1 mL min^{-1}, $\lambda = 210$ nm): $t_{major} = 6.63$ min, $t_{minor} = 7.20$ min.

2,2-Diethyl-3-(furan-2-yl)-4-nitrobutanal (**5b**) ^{1}H NMR (200 MHz, $CDCl_3$) δ 9.61 (s, 1H), 7.36 (dd, $J_1 = 0.6$ Hz, $J_2 = 1.8$ Hz 1H), 6.31 (dd, $J_1 = 1.8$ Hz, $J_2 = 3.2$ Hz, 1H), 6.21 (dd, $J_1 = 0.6$ Hz, $J_2 = 3.2$ Hz, 1H), 4.81–4.62 (m, 2H), 3.88 (dd, $J_1 = 4.9$ Hz, $J_2 = 10.0$ Hz, 1H), 1.72–1.51 (m, 4H), 0.91 (t, $J = 7.5$ Hz, 3H), 0.89 (t, $J = 7.5$ Hz, 3H). ^{13}C NMR (50 MHz, $CDCl_3$). δ 206.0, 149.4, 142.4, 110.4, 109.8, 75.2, 53.7, 41.0, 23.6, 22.9, 7.9, 7.6. IR ν_{max}: 2971, 2936, 2883, 1719, 1555. HRMS (ESI): m/z calcd for $C_{12}H_{21}N_2O_4$: 257.1496 $[M + NH_4]^+$; found 257.1501. HPLC (Chiralpak IA, i-propanol–heptane = 10/90, flow rate 1 mL min^{-1}, $\lambda = 210$ nm): $t_{minor} = 6.37$ min, $t_{major} = 6.92$ min.

1-(1-(Furan-2-yl)-2-nitroethyl)cyclohexanecarbaldehyde (**5c**)[42] ^{1}H NMR (200 MHz, $CDCl_3$) δ 9.59 (s, 1H), 7.37 (d, $J = 1.8$ Hz, 1H), 6.31 (dd, $J_1 = 1.8$ Hz, $J_2 = 3.2$ Hz, 1H), 6.20 (d, $J = 3.2$ Hz, 1H), 4.76–4.56 (m, 2H), 3.68 (dd, $J_1 = 4.8$ Hz, $J_2 = 10.2$ Hz, 1H), 2.11–1.16 (m, 10H). HPLC (Chiralpak IA, i-propanol–heptane = 10/90, flow rate 1 mL min^{-1}, $\lambda = 210$ nm): $t_{major} = 5.79$ min, $t_{minor} = 6.09$ min.

2-(1-(Furan-2-yl)-2-nitroethyl)-2-methylpentanal (**5d**) ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.55 (minor isomer) and 9.49 (major isomer) (s, 1H), 7.38 (major isomer) and 7.35 (minor isomer) (dd, $J = 0.6$, 1.8 and 0.6, 1.9 Hz, 1H), 6.32 (major isomer) and 6.30 (minor isomer) (dd, $J = 1.8$, 3.2 and 1.9, 3.2 Hz, 1H), 6.22 (major isomer) and 6.20 (minor isomer) (dd, $J = 0.6$, 3.2 and 0.6, 3.2 Hz, 1H), 4.76 (minor isomer) and 4.73 (major isomer) (dd, $J = 11.1$, 13.0 and 11.3, 12.8 Hz, 1H), 4.64 (minor isomer) and 4.55 (major isomer) (dd, $J = 3.8$, 13.0 and 3.6, 12.8 Hz, 1H), 3.99 (major isomer) and 3.90 (minor isomer) (dd, $J = 3.6$, 11.3 and 3.8, 11.1 Hz, 1H), 1.59–1.18 (m, 4H), 1.17 (major isomer) and 1.11 (minor isomer) (s, 3H), 0.91 (minor isomer) and 0.85 (major isomer) (t, $J = 7.2$ and 7.1, 3H) ^{13}C NMR 204.1, 149.7, 142.7, 110.6, 109.2, 75.3 (major isomer) and 74.7 (minor isomer), 51.5 (major isomer) and 51.1 (minor isomer), 42.7 (minor isomer) and 40.5 (major isomer), 37.6 (major isomer) and 36.6 (minor isomer), 17.1 (minor isomer) and 16.9 (major isomer), 16.5, 14.6 (minor isomer) and 14.4 (major isomer). IR ν_{max}: 2964, 2935, 2874, 1723, 1557. HRMS (ESI): m/z calcd for $C_{12}H_{21}N_2O_4$: 257.1496 $[M + NH_4]^+$; found 257.1500.

In vitro antimicrobial and cytotoxic assays

To test antimicrobial properties of synthesized γ-nitroaldehydes a range of bacterial strains from the American Type Culture Collection (ATCC) including *Micrococcus luteus* ATCC 379, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 27853 and *Candida albicans* ATCC 10259 in standard Kirby-Bauer disc diffusion assay. Minimal inhibitory concentrations (MIC) against *C. albicans* ATCC 10259 were determined in liquid culture in 96-well microtiter plate assay.[52]

Cytotoxicity of the compounds was assessed against human lung fibroblast MRC5 cell line obtained from ATCC using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay.[53] The MTT assay was performed after 24 h treatment with compounds three times in three replicates and the IC_{50} values (concentrations at which 50% cell growth inhibition occurred) were calculated in comparison to control (untreated cells) that were arbitrarily set to 100%.

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