Tetrahedron xxx (2015) 1–5

Contents lists available at ScienceDirect

Tetrahedron

journal homepage: www.elsevier.com/locate/tet

Synthesis of (1*R*,3*R*)-1-amino-3-methylcyclohexane by an enzyme cascade reaction

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ARTICLE INFO

Article history:
Received 9 October 2015
Received in revised form 3 November 2015
Accepted 5 November 2015
Available online xxx

Keywords:
Amine transaminase
Enoate reductase
Cascade reaction
Protein engineering
Small cyclic substrates

ABSTRACT

Amine transaminases (ATAs) are powerful enzymes for the synthesis of chiral amines. Although the request for amines with more than one chiral center is increasing, their synthesis is still challenging. Here we show a casacde reaction combining an enoate reductase (ERED) and an amine transaminase (ATA-VibFlu), which allows access to optically pure (1*R*,3*R*)-1-amino-3-methylcyclohexane. Because all known wildtype EREDs show a (*S*)-selectivity for 3-methylcyclohex-2-enone and the ATA-VibFlu only showed a modest enantioselectivity, different variants of EREDs and ATAs were investigated and suitable mutant enzymes were identified. In whole cell biocatalyses using the ERED YqjM Cys26Asp/Ile69Thr and the ATA-VibFlu Leu56Ile (1*R*,3*R*)-1-amino-3-methylcyclohexane was obtained at high optical purity (97% de).

1. Introduction

The interest for chiral amines increased dramatically in the last years. More than 80% of the top 200 current drugs contain amino functions[1] showing the importance of chiral amines as precursors for the pharmaceutical and fine-chemical industry.[2–4] Although several chemical methods to produce chiral amines have been developed,[5] the production by biocatalytic routes became more and more into the focus.[6–9,2,10] Compared to chemical routes biocatalysts mostly work at mild conditions, i.e., in aqueous phase at physiological pH, at ambient temperature and normal pressure. Furthermore, the high selectivity of enzymes can replace expensive chiral metal catalysts, which are required to achieve a chiral environment for chemical routes;[5] also the eco-efficiency can be increased.[11,12] One of the most famous example is the production of Sitagliptin, an antidiabetic compound. In this case the rhodium-catalyzed asymmetric enamine hydrogenation was replaced by biotransformation using an amine transaminase (ATA), which was optimized by protein engineering for this synthesis.[13,14] Indeed, transaminases became highly popular biocatalysts for the production of various chiral amines in the last decade.[2–4,15,16] Next to their ability to produce optically pure amines from a racemic mixture through kinetic resolution, they also can catalyze an asymmetric synthesis, which starting from a prochiral precursor allows a theoretical yield of 100%.

Transaminases are pyridoxal-5′-phosphate (PLP) dependent enzymes and catalyze the transfer of an amino group from an amino donor to an amino acceptor.[17–19] Depending on their substrate scope transaminases can be divided into α-, ω- and amine transaminases.[20] α-Transaminase convert substrates with a carboxylate in α-position to the carbonyl function, whereas for ω-transaminases at least one *C*-atom is in-between. Amine-transaminases substrates can lack completely the carboxylic group and hence ATA are the preferred enzymes to synthesize chiral amines.

ATA usually show high enantioselectivity[21–23] and this facilitates to synthesize compounds with multiple stereocenters by combining several enzymes in cascade reactions. Compared to chemical routes, the compatibility of different biocatalysts to each other is easier and they can be adapted to a certain range of reaction conditions.[12] Furthermore a missing selectivity of one enzyme for a certain chiral center can be overcome by the combination of various selective enzymes.[24]

Recently, we reported the synthesis of two 1-amino-3*S*-methylcyclohexane diastereomers by the combination of an enoate reductase and an amine transaminase.[25] The flavin-mononucleotide (FMN) containing and NAD(P)H-dependent enoate reductases (EREDs) catalyze the selective reduction of α,β-unsaturated ketones or aldehydes.[26] Whereas the wildtype enzyme of the ATA from *Vibrio fluvialis* (ATA-VibFlu) only showed a moderate selectivity for the conversion of racemic 3-methylcyclohexanone **2**, the enoate reductase Old Yellow Enzyme exhibited excellent (*S*)-selectivity for

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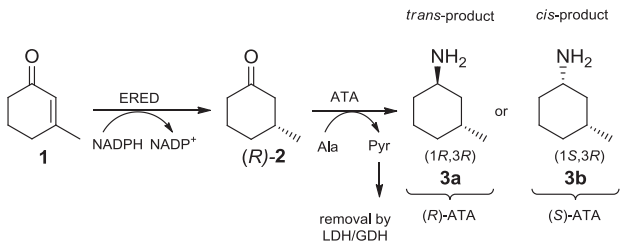
http://dx.doi.org/10.1016/j.tet.2015.11.005
0040-4020/

3-methyl-cyclohex-2-enone. 3DM guided protein engineering of the ATA-VibFlu led to two improved variants. 3DM is a structure-based database, which connects structure-guided sequence alignments with various other bioinformatic tools.[27,28] Sequences, structure information, protein ligands and mutational information from literature are integrated into this software. The ATA variants Leu56Ile and Leu56Val from *V. fluvialis* were generated guided by 3DM. In the application of the cascade reaction the diastereomeric purity of (1*R*,3*S*)-1-amino-3-methylcyclohexane was enhanced from 14% de to 66% de by the Leu56Val variant. In contrary, the cascade reaction of the OYE and the variant Leu56Ile resulted in 70% de of the (1*S*,3*S*)-diastereomer. The diastereoselectivity for the (1*R*,3*S*)-compound could be further enhanced by the addition of 30% DMSO to give 89% de. A recently published paper by Monti et al. described the synthesis of (1*R*,3*S*)- and (1*S*,3*S*)-1-amino-3-methylcyclohexane in a cascade reaction, too.[29] They used the Codexis ATA Screening kit (Codexis Inc., USA) and the OYE3 to achieve diastereomerically pure compounds. Nevertheless also they could only generate two out of the four diastereomers.

For the synthesis of the missing two diastereomers (**3a** and **3b**, Scheme 1) an enoate reductase with opposite enantioselectivity is required. The described wildtype enoate reductases all exhibit (*S*)-selectivity for **2**.[26,30,31] Only a mutant of the enoate reductase YqjM from *Bacillus subtilis*, described by Bougioukou et al., led to a switch in the selectivity.[32] The application of this mutant in a cascade reaction was already shown by Agudo et al. for the synthesis of 3-oxo-cyclohexane carboxylic acid methyl ester.[33] In this contribution, we aimed for using this enoate reductase to access the required two further diasteromers **3a** and **3b**.



Scheme 1. Synthesis of 1-amino-3-methylcyclohexane in a cascade reaction combining an enoate reductase (ERED) and an amine transaminase (ATA). The LDH/GDH enzymes are required to shift the equilibrium of the ATA-catalyzed reaction.

2. Results and discussion

2.1. Choice of appropriate enoate reductases

From literature data,[32] the two double mutants YqjM Cys26Asp/Ile69Thr and YqjM Cys26Asp/Ala104Trp were chosen as prime candidates. In addition, we also introduced these mutations into the xenobiotic reductase A (XenA) from *Pseudomonas putida* ATCC 17453[31] (corresponding positions: Cys25Asp, Ile66Thr and Ala101Trp) as this enzyme belongs as YqjM to the group of thermophilic like enoate reductases although they share only 38.4% sequence identity. Unfortunately, no soluble protein could be obtained. Nevertheless, the double mutants YqjM Cys26Asp/Ile69Thr and YqjM Cys26Asp/Ala104Trp could be generated via QuikChange mutagenesis and expressed in active form as reported.[32] Biocatalyses confirmed the (*R*)-selectivity described for both mutants, but as variant YqjM Cys26Asp/Ile69Thr was better expressed, all further experiments were performed with this variant.

2.2. Biocatalyses with the enoate reductase variant

In our earlier work purified EREDs were used,[25] but for YqjM it was reported that different tags impair the activity of the enzyme[34] and hence all reactions were now conducted as whole cell biocatalyses, which has the advantage that cofactor recycling is more easily facilitated. Whole cell biotransformation performed then using *E. coli* containing the YqjM Cys26Asp/Ile69Thr variant gave full conversion of 3-methylcyclohex-2-enone after 1.5 h (4 mmol L^{-1}) or after 4.5 h (10 mmol L^{-1}).

2.3. Cascade reaction of the YqjM variant and the amine transaminase VibFlu Leu56Ile

Thanks to our previous studies with different variants of the ATA-VibFlu using racemic 3-methylcyclohexanone **2**, the absolute configurations of the diastereomers of 1-amino-3-methylcyclohexane was already known[25] and hence we could easily determine the diastereomeric ratio between the (1*S*,3*R*)- and (1*R*,3*R*)-diastereomers after the cascade reaction: for all three variants of ATA-VibFlu (Leu56Ala, Leu56Ile and Leu56Val) the (1*R*,3*R*)-diastereomer was preferred over the (1*S*,3*R*)-diastereomer. As the Leu56Ile variant was the most stereoselective ATA in the synthesis of the (1*R*,3*R*)-diastereomer this variant was used in subsequent cascade reaction studies. The initial experiment was performed using *E. coli* whole cells harboring YqjM Cys26Asp/Ile69Thr to which the purified ATA-VibFlu Leu56Ile mutant was added. This biocatalysis resulted as expected in the formation of the (1*R*,3*R*)-1-amino-3-methylcyclohexane diastereomer and we were pleased to find that this resulted in excellent optical purity (97% de). The use of purified enzyme dramatically reduces the economic value of such a reaction and furthermore requires addition of LDH/GDH to shift the transaminase reaction towards product synthesis. Hence, we next transformed both plasmids encoding YqjM Cys26Asp/Ile69Thr and ATA-VibFlu Leu56Ile into *E. coli* BL21 (DE3) and after expression at 30 °C could obtain both enzymes in soluble form (Fig. 1).

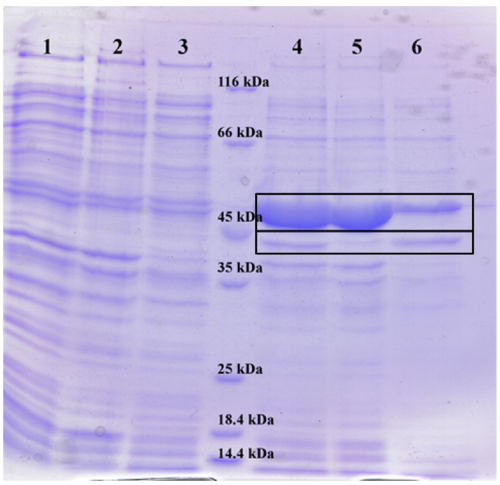


Fig. 1. SDS-PAGE of cultivation samples of the co-expression of the ERED YqjM Cys26Asp/Ile69Thr and the ATA VibFlu Leu56Ile. Lanes 1 and 4: crude extract, lanes 2 and 5: insoluble protein, lanes 3 and 6: soluble protein. Lines 1–3 show the protein content at the time of induction and lanes 4–6 show the protein content 5 h after induction. The upper frame shows the ATA, the lower frame the ERED.

Cascade reactions with resting cells containing the ERED as well as the ATA were performed in the presence or absence of LDH/GDH enzymes and the cofactor NADH. The comparison gave that the synthesis of 1-amino-3-methylcyclohexane was eight-fold higher after 60 h in the presence of the LDH/GDH system (83% conversion) compared to biocatalysis without it (11% conversion). This indicates that without this system pyruvate accumulates and the amine transaminase reaction is slowed down because of an unfavored

Please cite this article in press as: Skalden, L.; et al., Tetrahedron (2015), http://dx.doi.org/10.1016/j.tet.2015.11.005

equilibrium. Pyruvate is excreted by *E. coli* by an overflow production[35,36] and perhaps the added LDH/GDH system is able to reduce the excreted amount of pyruvate to balance its extracellular and intracellular concentration and thus enables a shift of the equilibrium to the desired asymmetric synthesis of the chiral amines. A similar system was described by Börner et al. for whole cell biocatalyses with ATAs to shift the reaction to the product site.[37] It was also observed that after 60 h biocatalysis in the presence of LDH/GDH, substrate **1** was nearly completely consumed, whereas the intermediate **2** was still present. The supplementation with LDH/GDH at this time point led to >99% conversion after 89 h. Similar to the first test with purified ATA, stereoselectivity of both enzymes was not altered and the product (1*R*,3*R*)-1-amino-3-methylcyclohexane was again obtained with 97% de.

2.4. Investigation of further amine transaminases to produce (1*S*,3*R*)-1-amino-3-methylcyclohexane

To identify amine transaminases, which prefer to produce the (1*S*,3*R*)-diastereomer instead of the (1*R*,3*R*)-diastereomer, both enantiomers of 3-methylcyclohexanone were docked with *YASARA*[38] into the crystal structure of the amine transaminase from *V. fluvialis* (pdb-code: 4E3Q). Additionally to Leu56, three further residues (small binding pocket: Phe19, Val153, large binding pocket: Ala228) were identified, which could influence the binding of *(R)*-3-methylcyclohexanone. These three residues were also targeted in other protein engineering approaches of this amine transaminase to alter its substrate scope.[39,22] With the help of 3DM suitable mutations on this positions were chosen, which led to 14 further variants in addition to the previous reported ones: Phe19Tyr/Cys/Val, Leu56/Met/Ser, Ala228Ie/Gly/Val/Cys/Ser/Thr and Val153Ala/Ile/Ser. All mutants could be expressed as soluble proteins. The screening against racemic 3-methylcyclohexanone showed unfortunately that none of these mutants produce (1*S*,3*R*)-1-amino-3-methylcyclohexane in excess compared to the (1*R*,3*R*)-diastereomer (Table 1).

Table 1
Composition of the diastereomers of 1-amino-3-methylcyclohexane produced by different variants of the ATA from *V. fluvialis* using *rac*-3-methylcyclohexanone as substrate

Variant	Conversion [%]	(1*R*,3*R*)	(1*S*,3*S*)	(1*S*,3*R*)	(1*R*,3*S*)
WT[a]	99	40	26	4	30
Phe19Cys[b]	16	34	50	4	12
Phe19Val[b]	2	36	38	2	24
Val153Ala[b]	12	55	29	2	14
Val153Ile[b]	15	45	44	3	8
Val153Ser[b]	3	41	42	7	10
Leu56Met[b]	8	43	44	5	8
Leu56Ser[b]	5	38	30	6	26
Ala228Thr[b]	4	42	39	10	9
Leu56Ile[a]	99	45.5	47	0.5	7
Leu56Val[a]	99	39	16	5	40

[a] Results from previous work with purified amine transaminase.[25]
[b] The screening against *rac*-3-methylcyclohexanone (10 mmol L^{-1}) was performed in deep-well plates (0.6 mL per well) with crude extract of the ATA variants for 78 h at 30 °C and 750 rpm.

Hence, the creation of an ATA variant showing the desired (1*S*,3*R*)-enantiopreference turned out to be not possible so far. The reason for this could be the orientation of the amino- and methyl-group at the cyclohexane ring. In the *cis*-diastereomer both substituents are positioned on one site of the cyclohexane ring, whereas in the *trans*-configuration, both are placed on opposite sites. These orientations seems to prevent the conversion of (*R*)-3-methylcyclohexanone to the (1*S*,3*R*)-diastereomer, although the synthesis of (1*R*,3*S*)-1-amino-3-methylcyclohexane did not suffer from this. Further investigations on the interplay between both binding pockets as well as mutations within each binding pocket of the ATA are hence required to explain and alter the selectivity of this amine transaminase from *V. fluvialis*.

3. Conclusion

In this work we have achieved the highly diastereoselective two-step synthesis of (1*R*,3*R*)-1-amino-3-methylcyclohexane by the combination of the enoate reductase variant YqjM Cys26Asp/Ile69Thr and the amine transaminase variant Leu56Ile from *V. fluvialis*. Mutagenesis studies for ATA-VibFlu to produce also the missing (1*S*,3*R*)-diastereomer unfortunately failed pointing out the complex design of enzyme stereopreference.

4. Experimental section

4.1. Materials

All chemicals were purchased from Fluka (Buchs, Switzerland), Sigma (Steinheim, Germany), Merck (Darmstadt, Germany), VWR (Hannover, Germany), or Carl Roth (Karlsruhe, Germany) and were used without further purification unless otherwise specified. Polymerases were obtained from New England Biolabs GmbH (NEB, Beverly, MA, USA) and primers were ordered from Invitrogen (Life Technologies GmbH, Darmstadt, Germany).

4.2. Bacterial strains and plasmids

E. coli TOP10 [F'lacIq, Tn10(TetR) mcrA D(mrr-hsdRMS-mcrBC) F80 LacZDM15 DlacX74 recA1 araD139 D(ara leu)7697 galU galK rpsL (StrR) endA1 nupG] was obtained from Invitrogen (Carlsbad, CA, USA). *E. coli* BL21 (DE3) [fhuA2 [lon] ompT gal (l DE3) [dcm] DhsdS] was purchased from New England Biolabs (Beverly, MA, USA). The plasmid pET24b bearing the gene encoding the ATA from *V. fluvialis* (accession no. F2XBU9) was kindly provided by Prof. Byung Gee Kim (Seoul National University, South-Korea).

4.3. Cloning

The enoate reductase variants were generated via QuikChange PCR with specific primers for each variant:

XenA:

Cys25Asp fw:5′−CAT TCC GCC CGA TTG CCA GTA CAT G−3′
Cys25Asp rv:5′−CAT GTA CTG GCA ATC GGG CGG AAT G−3′
Ile66Thr fw:5′−GAA GGG CGC ACC ACC CCT GG−3′
Ile66Thr rv:5′−CCA GGG GTG GTG CGC CCT TC−3′
Ala101Trp fw:5′−GCA TCC AGA TTT GGC ACG CCG−3′
Ala101Trp rv:5′−CGG CGT GCC AAA TCT GGA TGC−3′

YqjM:

Cys26Asp fw:5′−CATGTCGCCAATGGATATGTATTCTTCTC−3′
Cys26Asp rv:5′−GAGAAGAATACATATCCATTGGCGACATG−3′
Ile69Thr fw:5′−CCC TCA AGG ACG AAC CAC TGA CCA AGA C−3′
Ile69Thr rv:5′−GTC TTG GTC AGT GGT TCG TCC TTG AGG G−3′
Ala104Trp fw:5′−CGG CAT TCA GCT TTG GCA TGC CGG ACG−3′
Ala104Trp rv:5′−CGT CCG GCA TGC CAA AGC TGA ATG CCG−3′

The plasmid (2 μL) was mixed with Pfu^+ buffer (5 μL), dNTPs (1.5 μL), Pfu^+ polymerase (0.5 μL), primers (2 μL of each) and distilled water (35.5 μL). The PCR program included the following temperature steps: hold at 95 °C for 5 min, afterwards 25 cycles of

Please cite this article in press as: Skalden, L.; et al., Tetrahedron (2015), http://dx.doi.org/10.1016/j.tet.2015.11.005

the following: hold at 95 °C for 45 s, 53 °C for 45 s, 72 °C hold for 7.5 min. Finally hold at 72 °C for 10 min.

All ATA-VibFlu variants were generated via MegaWhop PCR with specific forward or reverse primer for each variant. The other corresponding primer was either the T7 forward or T7 reverse primer.

Phe19Cys:5′–G CTC TAT GGT TGC ACC GAC ATG C–3′
Phe19Val:5′–G CTC TAT GGT GTG ACC GAC ATG C–3′
Phe19Tyr:5′–G CTC TAT GGT TAT ACC GAC ATG C–3′
Leu56Ser:5′–GCC AAC TCG GGC AGC TGG AAC ATG G–3′
Leu56Met:5′–CGC AAC TCG GGC ATG TGG AAC ATG G–3′
Val153Ile:5′–GCC TAT CAC GGC ATC ACC GCC GTT TC–3′
Val153Ser:5′–GCC TAT CAC GGC AGC ACC GCC GTT TC–3′
Val153Ala:5′–GCC TAT CAC GGC CGT ACC GCC GTT TC–3′
Ala228Cys:5′–CGG TGA TGG GCT GCG GCG GCG TG–3′
Ala228Ile:5′–GTG ATG GGC ATC GGC GGC GTG–3′
Ala228Gys:5′–CG GTG ATG GGC GGC GGC GGC GTG–3′
Ala228Ser:5′–CG GTG ATG GGC AGC GGC GGC GTG–3′
Ala228Thr:5′–CG GTG ATG GGC ACC GGC GGC GTG–3′
Ala228Val:5′–CG GTG ATG GGC GTG GGC GGC GTG–3′

The plasmid (4 μl) was mixed with Pfu$^+$ buffer (5 μL), dNTPs (1.5 μL), Pfu$^+$ polymerase (0.5 μL), specific primer (1 μL), T7 primer (2 μL) and distilled water (35.5 μL). The PCR program included the following temperature steps: hold at 95 °C for 5 min, afterwards 30 cycles of the following: hold at 95 °C for 45 s, 54.8 °C for 45 s, 72 °C hold for 6 min. Finally hold at 72 °C for 10 min. The PCR products (8 μL) were mixed with 10x Pfu$^+$ buffer (5 μL), plasmid (2 μL), dNTPs (1 μL), Pfu$^+$ polymerase (1 μL) and distilled water (33 μL). The following program was used: hold 68 °C for 5 min, hold 95 °C for 1 min, 10 cycles of the following three steps: 95 °C for 30 s, 53 °C for 30 s and 68 °C for 7 min. Afterwards 10 cycles of the following three steps were made: 95 °C hold for 30 s, 55 °C hold for 30 s and 68 °C hold for 11 min.

4.4. Cultivation, expression and purification

The cultivation and expression of the EREDs and the ATAs were performed as described elsewhere.[32,31,25] The purification of the ATA VibFlu Leu56Ile was performed as described previously.[25] After the co-transformation of the plasmids bearing the genes encoding ERED or ATA (each 1 μL) in chemo-competent *E. coli* Bl21 (DE3) cells, the cultivation was performed in TB media containing 0.1 mg mL^{-1} ampicillin and 0.05 mg mL^{-1} kanamycine. The cells were incubated at 37 °C until an OD_{600} of 0.7 was reached. After the induction with IPTG (100 μmol L^{-1}) the cells were further incubated at 30 °C and 180 rpm for 5 h before they were harvested by centrifugation for 15 min at 4 °C. The cells were used immediately for biotransformations.

4.5. Activity measurements

The activity of the amine transaminases was determined by the acetophenone-assay.[40] The activity of the EREDs were determined via biocatalyses and GC analysis.

4.6. Biocatalyses

The total volume was 0.9 mL. Biocatalysis was performed in sodium phosphate buffer (50 mmol L^{-1}, pH 7.5) with substrate (10 mmol L^{-1}), glucose (3 mmol L^{-1}) and whole cells or crude extract (0.2 g wet weight mL^{-1}). The substrate solution was prepared as a stock solution (1 mol L^{-1}) in DMSO. The reaction was incubated at 30 °C at 750 rpm. For whole cell biocatalyses containing the ERED and the ATA, L-alanine (200 mmol L^{-1}), NADH (1 mmol L^{-1}), GDH (0.9 U) and LDH (0.15 U) was added. Biocatalyses with ATA and *rac*-**2** had a total volume of 0.6 mL and were performed in deep well plates. The biocatalyses contained: the crude extract of the amine transaminases, GDH (0.9 U), LDH (0.15 U), L-alanine (200 mmol L^{-1}), glucose (1 mmol L^{-1}), substrate (10 mmol L^{-1}) and NADH (1.1 mmol L^{-1}) dissolved in sodium phosphate buffer (50 mmol L^{-1}, pH 7.5) including PLP (0.1 mmol L^{-1}). The substrate solutions were prepared as stock solution (1 mol L^{-1}) in DMSO. The reactions were incubated for 78 h at 30 °C at 750 rpm. Biocatalyses samples (300 μL) were mixed with NaOH (150 μL of 1 mol L^{-1}) and extracted at first with ethylacetate (300 μL) and then with hexane (150 μL). The organic phases were combined and dried with anhydrous sodium sulfate before GC analysis was performed.

4.7. Docking studies

The docking studies were performed with *YASARA*[38] using the structure of the ATA from *V. fluvialis* (pdb-code 4E3Q). (*R*)- and (*S*)-3-methylcyclohexanone were alternatively docked into the active site. The chosen simulation cell was defined to be 18×17×18 Å^3. All residues of the active site and the active site loop were included.

4.8. Analytics

The determination of the conversion, the ratio of the diastereomers or enantiomers were identified via GC as described previously.[25] The derivatisation of the samples was performed with 5 μL trifluoroacetic acid anhydride.

Acknowledgements

We thank the European Union (KBBE-2011-5, grant no. 289350) and the DFG (grant no. Bo1862/6-1) for financial support. We are grateful to Prof. Byung-Gee Kim (Seoul National University, Korea) for the gene encoding the ATA from *V. fluvialis*.

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Please cite this article in press as: Skalden, L.; et al., Tetrahedron (2015), http://dx.doi.org/10.1016/j.tet.2015.11.005