Broadening the scope of Baeyer-Villiger Monooxygenase Activities toward α,β-Unsaturated Ketones: A promising route to chiral enol-lactones and ene-lactones

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Electronic Supplementary Information

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1. BVMO enzymes

BVMO$_{Ocean}$ (UniProtKB/TrEMBL accession number: A3U3H1): the gene encoding for this enzyme was obtained from *Oceanicola batsensis* DSM-15984. The corresponding protein (545 aa, 60.6 kDa) was originally automatically annotated as "Flavin-containing monooxygenase FMO:FAD dependent oxidoreductase".

BVMO$_{Parvi}$ (UniProtKB/TrEMBL accession number: A7HU16): the gene encoding for this enzyme was obtained from *Parvibaculum lavamentivorans* DSM-13023. The corresponding protein (544 aa, 61.8 kDa) was originally automatically annoted as "Cyclohexanone Monooxygenase".

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tr|A3U3H1|A3U3H1_9RHOB      -------MNIQTENTKTVGADFDAVVIGAGFGGLYAVHKLRNEQGLNVRG 43
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tr|A3U3H1|A3U3H1_9RHOB      YDSASDVGGTWWWNRYPGALSDTESYVYRYSFDKELLRKGRWKTRYLTQP 93
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tr|A3U3H1|A3U3H1_9RHOB      EILEYMNEVADHLDLRRSYKFDTKVDGAHYNEKTGLWNVITDSGETVTAK 143
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sp|Q937L5|CPNB_COMTE        FVIANLGFGASPSTPNVDGIETFKGQWYHTALWPQEGVNMAGKRVAIIGT 198
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sp|P12015|CYMO_ACISP        FLITALGLLSAPNLPNIKGINQFKGELHHTSRWP-DDVSFEGKRVGVIGT 184
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sp|P12015|CYMO_ACISP        GSTGVQVITAVAPLAKHLTVFQRSAQYSVPIGNDPLSEEDVKKIKDNYDK 234
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                             *   .  **    :* :.*    :*:* :*:** * .      .***
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sp|Q937L5|CPNB_COMTE        EQWYYEIFNQNNVTLVDVNETPVLRITEKGIVTAEG-EAEFDLIVFATGF 396
tr|A3U3H1|A3U3H1_9RHOB      GNNYYEVYNRDNVTLADVKADPIAEFTPNGIRLESGEEHELDIVIFATGF 388
sp|P12015|CYMO_ACISP        DSGYYNTFNRDNVRLEDVKANPIVEITENGVKLENGDFVELDMLICATGF 380
                             . **: :*::** * **.  *: .:. :*:   .*   *:*::: ****
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tr|A3U3H1|A3U3H1_9RHOB      DAVDGNYVKMDLRGRGGVTMRDTWKEGPLGYLGMMEVDFPNFFMILGPNG 438
sp|P12015|CYMO_ACISP        DAVDGNYVRMDIQGKNGLAMKDYWKEGPSSYMGVTVNNYPNMFMVLGPNG 430
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sp|P12015|CYMO_ACISP        P--FTNLPPSIESQVEWISDTIQYTVENNVESIEATKEAEEQWTQTCANI 478
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tr|A7HU16|A7HU16_PARL1      AGMTLFPRADSWYMGANIPGKPRQLLNFP-GVPIYMDQCNTAAAKDYEGF 541
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tr|A3U3H1|A3U3H1_9RHOB      ADMTLFPKAESWIFGANIPGKKNAVMFYMAGIGNYRNAISAVKEEGYT-- 534
sp|P12015|CYMO_ACISP        AEMTLFPKAQSWIFGANIPGKKNTVYFYLGGLKEYRTCASNCKNHAYEGF 528
                               :***:*.**  *:*****      :  *:  *    .    . *
tr|A7HU16|A7HU16_PARL1      VLD------------ 544
sp|Q937L5|CPNB_COMTE        SLAS----------- 550
tr|A3U3H1|A3U3H1_9RHOB      --SLIRDRTAEKV-- 545
sp|P12015|CYMO_ACISP        DIQLQRSDIKQPANA 543
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Figure 1: CLUSTALW2 Multiple sequence alignment of BVMO$_{Ocean}$ and BVMO$_{Parvi}$ with CHMO$_{Acineto}$ (P12015) and CPMO$_{Coma}$ (Q937L5). All enzymes contain the conserved BVMO motifs [A/G]GxWxxxx[F/Y]P[G/M]xxxD (red frame)[1] and FXGXXXHXXXW[P/D] (blue frame)[2].

Table 1: Sequence identity (%) between $BVMO_{Ocean}$, $BVMO_{Parvi}$, $CHMO_{Acineto}$ (P12015) and $CPMO_{Coma}$ (Q937L5) calculated with CLUSTALW2.

	$BVMO_{Ocean}$	$BVMO_{Parvi}$	$CHMO_{Acineto}$	$CPMO_{Coma}$
$BVMO_{Ocean}$	100	38	58	36
$BVMO_{Parvi}$	38	100	41	53

2. Materials

Luria-Bertani (LB) and Terrific Broth (TB) media were obtained from MP Biomedicals. Ampicillin and kanamycin were obtained from Sigma-Aldrich.

Cyclopentanone, cyclohexanone (99.8%), cycloheptanone, caprolactone, alpha-lactose, 2-cyclopenten-1-one (98%), 2-cyclohexen-1-one (98%), 2-methyl-cyclohexen-1-one, 2-methyl-cyclohexanone, 3-methyl-cyclohexanone, 4-methyl-cyclohexanone and 2-cyclohepten-1-one were obtained from Sigma-Aldrich. 3-methyl-cyclopentenone and 3-methyl-cyclohexenone were obtained from Alfa Aesar. 2-cyclohexen-1-one was purified by chromatography on silica gel to remove traces of cyclohexanone.

NMR spectra were recorded in $CDCl_3$ on a 300-MHz Bruker Avance III Nanobay instrument. Chemical shift (δ) were given in ppm relatively to chloroform or TMS.

GC analyses were carried out on a Shimadzu GC-14A gas chromatograph equipped with an Equity™-5 column (Supelco). GC-MS analyses were performed using a Shimadzu QP-2010/SE device equipped with an ion trap mass detector and the same column as above. HRMS values were obtained using a mass spectrometer QStar Elite (Applied Biosystems SCIEX).

Chiral GC analyses were carried out with an Hydrodex γ-DiMOM column (Macherey-Nagel) or Chirasil-DEX CB column (Varian).

3. Constructions of *E. coli* BL21(DE3) strains

Table 2: List of strains used in this work

Name	Relevant characteristics	References
BL21(DE3)	F^- *ompT* *hsd*S_B(r_B^-, m_B^-) *gal dcm* (DE3)	Novagen
BW25113	*rrnB3* Δ*lacZ4787 hsdR514* Δ(*ara*BAD) Δ(*rha*BAD)568 *rph-1*	Ref [3]
BW25113Δ*nem*A	BW25113 *ΔnemA::kan*^{R}	Ref [4]
BL21(DE3)Δ*nem*A	BL21(DE3)Δ*nemA*::*kan*^{R}	This work

a) Construction of *E. coli* BL21(DE3) expressing BVMO

Genes selected for the large scale screening of the collection, were cloned according ligation independent cloning method[5]. Specific extensions were added to the primers for cloning into our plasmid pET22b(+) (Novagen) modified for ligation independent cloning. Oligonucleotides were from Sigma-Genosys. The *bvmo* genes corresponding to A3U3H1 enzyme from *O. batsensis* DSM-15984 and A7HU16 from *P. lavamentivorans* DSM-13023 selected from the large-scale screening were re-cloned with the forward primers introducing a hexahistidine sequence in the proteins after the initial methionine for purification purposes.

These genes were cloned in the same pET22b(+) modified for ligation independent cloning. The plasmid were then introduced into *E. coli* BL21 (DE3) plysE strains (Invitrogen) for over-expression.

b) Construction of *E. coli* BL21(DE3)Δ*nem*A

BW25113Δ*nem*A deletion mutant strains was obtained from the Keio collection (http://ecoli.aist-nara.ac.jp/).[3] P1 transduction of a Δ*nem*A::kan^R allele from BW25113Δ*nem*A in the BL21(DE3) strain was performed according to the classical procedure described by Miller[6] and modified by My *et al.*[7] The phage was grown on a strain containing the elements to be moved, and the resulting phage lysate, containing bacterial DNA as well as phage DNA, was used to infect a second recipient strain. A genetic recombination, catalyzed by enzymes of the recipient strain, incorporated the bacterial fragments into the recipient chromosome. Concisely, a lysate was prepared with the donor strain. Cells (equivalent to 0.9 g dry cells) in 5 mM $CaCl_2$ were placed in contact with P1 lysate from the WT strain until lysis completion. For the transduction step, the recipient strain (equivalent to 0.3 g dry cells) in 5 mM $CaCl_2$ was mixed with the "donor lysate" for 20 min at 37°C. Cells were washed twice with 5mM citrate solution. Finally BL21(DE3)Δ*nem*A strain was selected onto kanamycin plates (25 $mg.L^{-1}$) supplemented with 2mM citrate.

c) Construction of expressing BVMO *E. coli* BL21(DE3)Δ*nem*A

20ng of plasmid (pet22b-*bvmo* gene) were added into 100 µL of competent cells BL21(DE3)Δ*nem*A prepared as usual using a $CaCl_2$ solution. After 1 hour at 4°C, the solution was warmed 30 s at 42°C, replaced at 4°C for 5 min. After addition of 900 µL of LB medium, the solution was shaken (200 rpm) at 37°C for 1 hour, then spread on plate containing ampicillin (50 $mg.L^{-1}$) and kanamycin (25 $mg.L^{-1}$) and left at 37°C overnight.

$BVMO_{Ocean}$ ($BVMO_{Parvi}$) expression levels in the initial BL21(DE3) and BL21(DE3)Δ*nem*A strains were similar as shown by the activity of the cells measured on cyclohexanone (77 and 83; respectively 63 and 72 $U.g^{-1}$ dry cells).

4. Cell growth

Wild-type and recombinant *E. coli* BL2(DE3) strains were grown at 23°C on Luria-Bertani or Terrific Broth supplemented with kanamycin (50 mg. L^{-1}) when necessary. The plasmids were maintained with ampicillin (100 $mg.L^{-1}$). Enzyme production was induced by lactose (2% w/v) when OD_{600nm} was around 0.8. Cells were used after 20h of growth.

5. Enzyme activity assays

a) On crude extracts

$BVMO_{Parvi}$ and $BVMO_{Ocean}$ were produced by *E. coli* BL21(DE3)Δ*nem*A strains. Cells were sonicated in Tris-HCl buffer solution (50mM, pH 7.5). NADPH (40µM), substrate (10µL, 1M in acetonitrile) and crude cell extract (100µL) were added to Tris-HCl buffer solution (890 µl, 50 mM, pH 8.5). Enzyme activities (Table 2) were measured by monitoring

NADPH consumption at 340 nm. Sample without substrate was used as a blank. Enzyme concentration was estimated using the FAD assay described by Riebel.[1]

Table 3. Enzymatic activities of cell extracts based on NADPH comsumption

Substrate	**BVMO**$_{Parvi}$ 10^6.U/mole enzyme	**BVMO**$_{Ocean}$ 10^6.U /mole enzyme	**CHMO**$_{Acineto}$ U/mole enzyme
Cyclobutanone	38	34	13
Cyclopentanone	39	30	38
2-Cyclopentenone	24	<1	0
3-methyl-2-cyclopenten-1-one	18	1	0
Cyclohexanone	29	21	56
2-Cyclohexenone	12	11	0
2-methyl-cyclohexanone	23	24	36
3-methyl-cyclohexanone	28	23	43
3-methyl-2-cyclohexen-1-one	32	2	0
4-methyl-cyclohexanone	31	23	58
4-methyl-2-cyclohexen-1-one	7	23	1
2-Cycloheptenone	9	19	nd
Cyclodecanone	0	3	nd
Bicyclo[3.2.0]hept-2-en-6-one	30	15	54
Norcamphor	33	10	nd
1-indanone	35	9	nd
acetophenone	30	2	nd

b) On purified enzymes

BVMO$_{Parvi}$ and BVMO$_{Ocean}$ were produced by *E. coli* BL21(DE3) strains. They were purified by loading the crude extract onto a Ni-NTA column (Qiagen) according the manufacturer's instructions. Enzyme activities (Table 1) were measured as previously described by monitoring NADPH consumption at 340 nm.

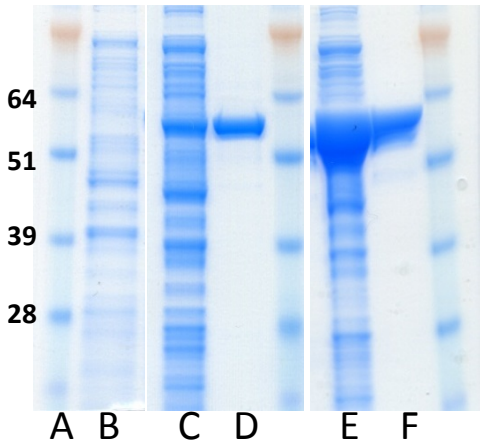


Figure 2: Protein expression (with and without lactose induction) and purification profile of both BVMOs (SDS-Page).

A:Protein ladder, B: *E. coli* BL21(DE3), C: BVMO$_{Parvi}$ *E. coli* BL21(DE3); D: purified BVMO$_{Parvi}$; E: BVMO$_{Ocean}$ *E. coli* BL21(DE3); F: purified BVMO$_{Ocean}$.

Table 4. Enzymatic activities of purified enzymes based on NADPH comsumption

Substrate	$BVMO_{Parvi}$ mU/mg P	$BVMO_{Ocean}$ mU/mg P
Bicyclo[3.2.0]hept-2-en-6-one	314	239
Cyclopentanone	nd	392
Cyclohexanone	352	347
2-Cyclopentenone	**192**	**0**
2-Cyclohexenone	**170**	**55**
2-Cycloheptenone	**30**	**235**
Norcamphor	193	136
1-indanone	230	44
Acetophenone	132	0

6. Chemical Synthesis

a) Synthesis of 4-methyl-2-cyclohexen-1-one (5g)

As previously described,[8] a catalytic amount of *t*-BuOK (0.05 equiv) was added to a stirred solution of ethyl-3-oxobutanoate (14 mmol) and methacryaldehyde (1eq) in *t*-BuOH (1 M) at 0°C. The reaction mixture was maintained to 0°C and stirred for 30 min before another addition of 0.2 equiv of *t*-BuOK. The mixture was then heated under reflux for 20 h. then quenched with HCl solution (1M, 10 mL), diluted with ether (80 mL), washed with NaOH solution (1 M, 3x20 mL) and brine (2x20 mL). The separated organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography to give **5g** (408 mg, 26% yield) as a pale yellow oil.
4-methyl-cyclohexen-2-one **5g**: 1H NMR (300 MHz, $CDCl_3$) δ 6.8 (dq, *J*= 10, 1Hz, 1H), 5.9 (dd, *J* =10, 2Hz, 1H), 2.4 (m, 2H), 2.1 (m, 2H), 1.5 (m, 1H), 1.3 (m, 1H), 1.2 (d, *J* =8Hz, 3H)
^{13}C NMR (75 MHz, $CDCl_3$) δ 199, 155.9, 128.3, 36.6, 30.8, 30.7, 19.9

b) General procedure for chemical Baeyer-Villiger oxidation

To a solution of 70-75% pure mCPBA (4 mmol) and $NaHCO_3$ (5 mmol) into 5 mL of CH_2Cl_2 were added dropwise cycloalkanone or cycloalkenone (2 mmol) at 0°C. The mixture was stirred overnight then quenched with 10 mL of an aqueous Na_2SO_3 solution (10%). The mixture was extracted by CH_2Cl_2 (3x10 mL) and washed with a saturated $NaHCO_3$ solution (10x2 mL) and brine (10 mL). After drying over anhydrous $MgSO_4$ and concentration under reduced pressure, the crude product was purified by silica gel column chromatography.

7. Preparative scale Biotransformations

Baffled flasks were preferred to fermentors to avoid the stripping of volatile compounds under the action of high air flow.

Wild-type and recombinant *E. coli* BL21(DE3) strains were grown at 23°C in 2L flasks filled with 300 mL of Terrific Broth supplemented with ampicillin and kanamycin when necessary. If needed, enzyme production was induced by lactose (2% w/v) when OD_{600nm} was around 0.8. After 20h of growth, cells were used directly for whole-cell biotransformation. Ethanolic solution of enone (final concentration 3mM) was added to the culture medium. Aliquots of the reaction mixture were extracted by AcOEt containing decane, undecane or dodecane as internal standard and analyzed by GC and GC-MS.

NMR analyses were performed on purified lactones obtained from preparative scale experiments (300mL). After continuous CH_2Cl_2 extraction from biotransformation medium, the compounds were purified by flash chromatography. The eluent was a mixture of pentane/AcOEt (1/1) except for **4c** for which a mixture of CH_2Cl_2/AcOEt (99/1) was used. The products were identified by comparison of NMR and GC-MS spectra with those of chemically synthesized samples and/or previously published data.

Table 5: Reductase activity based on whole-cell biotransformation of cycloalkenones **1a-c**

E. coli strains[a]	Reductase activity against ketone (U. g^{-1} dry cells)[b]		
	1a	**1b**	**1c**
BL21(DE3)	6	22	13
BW25113Δ*nem*A	1	7	6

[a] Cell concentration : 3.6 g^{-1} dry cells.L^{-1}. [b] Activity determined by GC analysis of resulting saturated ketones using decane or undecane as internal standard. nd: not determined.

Table 6: Biotransformations of methylated cycloalkenones **5a-c** by *E. coli* BL21(DE3)Δ*nem*A strain producing $BVMO_{Ocean}$ and by $CHMO_{Acineto}$

Ketone[a]	Time	Residual Ketone **5**		Lactone **6**		Lactone **7**		E	ref
		Yield[b]	*ee* (*abs conf*)[c]	Yield[b]	ee (*abs conf*)[c]	Yield[b]	ee (*abs conf*)[c]		
5a	0.5	70 %	*29% ee (R)*	24%	*56% ee (S)*	0	_	5	
					(S)			*10*	[9]
5b	0.5	0	-	50%	*98% ee (R)*	48%	*98% ee (S)*	-	
					98% ee (*R*)		98% ee (*S*)		[10]
5c	2	0	-	93%	*98% ee (S)*	0	-	-	
					>98% (*S*)				[11]

[a] Cell concentration: 4.8 g^{-1} dry cells.L^{-1}. [b] Yields: determined by GC analysis using undecane or dodecane as internal standard. [c] Ees: determined by GC analysis on chiral columns. Absolute configurations: assigned by comparison of enantiomer retention times with those of authentic compounds from CHMO mediated biotransformations. In blue, previously published CHMO results.

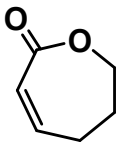
Table 7: Biotransformations of methylated cycloalkenones **5a-c** by *E. coli* BL21(DE3)*Δnem*A strain producing $BVMO_{Parvi}$

Ketone[a]	Time	Residual ketone **5**		Lactone **6**		Lactone **7**		E[d]	Ref
		yield[b]	ee (*abs conf*)[c]	yield[b]	ee (*abs conf*)[c]	yield[b]	ee (*abs conf*)[c]		
5a	0.5	78%	*14% ee(S)*	21%	*23% ee(R)*	0	-	3	
5b	0.5	79%	*33% ee(R)*	20%	*88% ee(S)*	0	-	15	
					98% (*R*)			>100	[10]
5c	2	0	-	92%	*68% ee (R)*	0	-	-	
					46% ee (*R*)				[12]

[a] Cell concentration was 4.8 g^{-1} dry cells.L^{-1}. [b] Yields were determined by GC analysis using undecane or dodecane as internal standard. [c] ees were determined by GC analysis on chiral columns. Absolute configurations were assigned by comparison of enantiomer retention times with those of authentic compounds prepared by CHMO mediated biotransformations. [d] E, the enantiomeric ratio, was calculated from ees. In blue, previously published CPMO results

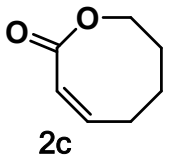
8. Compounds identification

6,7-dihydro-2(5*H*)-oxepinone (2b) [13, 14, 15]: Biotransformation of 84 mg of cyclohexenone **1b** with 300 mL of expressing $BVMO_{Ocean}$ BL21(DE3)Δ*nem*A cells afforded **2b** (47mg, 48% yield) as a pale yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.4 (dt, *J*= 12, 4.4Hz, 1H), 6 (dt, *J*=12, 2Hz, 1H), 4.3 (t, *J*= 4.6Hz, 2H), 2.5 (m, 2H), 2.1 (m,2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 169, 144, 122, 67, 30, 27. GC-MS: *m*/*z* = 112 (M^+) 96, 84, 71, 54.



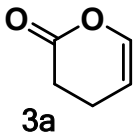
2b

5,6,7,8-tetrahydro-2*H*-oxocin-2-one (2c) [14,15]**:** Biotransformation of 101 mg of cycloheptenone **1c** with 300 mL of expressing $BVMO_{Ocean}$ BL21(DE3)Δ*nem*A cells afforded **2c** (71mg, 62% yield) as a pale yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.3 (dt, *J*= 13, 5Hz, 1H), 5.7 (dt, *J*= 13, 2Hz, 1H), 4.4 (t, *J*= 5.5Hz, 2H), 2.4 (m, 2H), 1.9 (m, 2H) 1.7 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.4, 141.8, 117, 67.4, 30.6, 30, 18.9. GC-MS: *m*/*z* = 126 (M^+) 108, 98, 81, 68, 55.



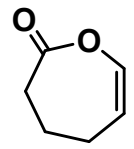
2c

3,4-dihydro-2*H*-pyran-2-one (3a) [16]: Biotransformation of 74 mg of cyclopentenone **1a** with 300 mL of expressing $BVMO_{Parvi}$ BL21(DE3)Δ*nem*A cells afforded **3a** (57mg, 64% yield) as a pale yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.6 (dt, *J*= 6, 1.5Hz, 1H), 5.3 (q, *J*= 6Hz, 1H), 2.6 (t, *J*= 17.5Hz, 2H), 2.3 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.3, 141.7, 105.4, 28.6, 18.5; GC-MS: *m*/*z* = 98 (M^+) 70, 55.



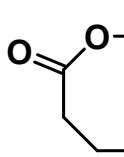
3a

4,5-dihydro-2(3*H*)-oxepinone (3b) [16]: Biotransformation of 84 mg of cyclohexenone **1b** with 300 mL of expressing $BVMO_{Parvi}$ BL21(DE3)Δ*nem*A cells afforded **3b** (65mg, 66% yield) as a pale yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.3 (dt, *J*= 6.5, 1.3Hz, 1H), 5.3 (q, *J*= 6.5Hz, 1H), 2.7 (t, *J*= 6.5Hz, 2H), 2.3 (m, 2H), 2.1 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.3, 140.1, 113.3, 33.1, 24.7, 23.3; GC-MS: *m*/*z* = 112 (M^+) 98, 82, 67, 55.



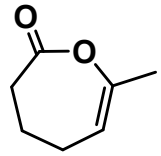
3b

3,4,5,6-tetrahydro-*2H*-oxocin-2-one (3c)[16]: Biotransformation of 100 mg of cycloheptenone **1c** with 300 mL of expressing $BVMO_{Parvi}$ BL21(DE3)Δ*nem*A cells afforded **3c** (10mg, 9% yield) as a pale yellow oil. Because a small difference of chemical shifts was observed with literature data, we confirmed the identification of **3c** by 2D NMR analyses (COSY, HMBC, HSQC) and comparison with authentic sample synthesized by chemical BV oxidation (MCPBA, $NaHCO_3$). ^{1}H NMR (300 MHz, $CDCl_3$) δ δ 6.3 (dt, *J*= 6.2, 1Hz, 1H), 5.2 (dt, *J*= 8.5, 6.2Hz, 1H), 2.5 (m, 2H), 1.9 (m, 2H), 1.8 (m, 2H), 1.5 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.4 (C), 139.4 (C<u>H</u>-O-), 118.5(C<u>H</u>-CH_2-), 31.2 (C<u>H</u>$_2$-C=O), 25.5 (C<u>H</u>$_2$-C=C-), 24.6 (C<u>H</u>$_2$-CH_2-C=O), 22.7 (C<u>H</u>$_2$-CH_2-C=C-). GC-MS: *m*/*z* = 126 (M^+) 112, 96, 81, 67, 55.



3c

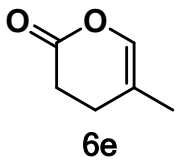
7-methyl-4,5-dihydrooxepin-2(3H)-one (6d): Biotransformation of 100 mg of 2-methyl-cyclohexenone **5d** with 300 mL of expressing $BVMO_{Parvi}$ BL21(DE3)Δ*nem*A cells afforded **6d** (68 mg, 60% yield) as a pale yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ ; 5.3 (dt, *J*= 7, 1Hz, 1H), 2.6 (t, *J*= 7Hz, 2H), 2.1(m, 4H), 1.8 (d, *J*= 1Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ ; 172.7, 14.9, 109, 32.4, 27.2, 21.9,



6d

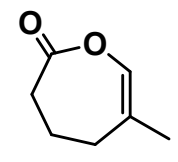
19.5. GC-MS: *m*/*z* = 126 (M^+) 112, 96, 84,79, 67, 55. HRMS: calcd for $C_7H_{10}O_2$: 127.0754 $[M+H]^+$, found: 127.0751.

5-methyl-3,4-dihydro-2H-pyran-2-one (6e)[17]**:** Biotransformation of 84 mg of 3-methyl-cyclopentenone **5e** with 300 mL of expressing $BVMO_{Parvi}$ BL21(DE3)Δ*nem*A cells afforded **6e** (59 mg, 52% yield) as a pale yellow oil. ^1H NMR (300 MHz, $CDCl_3$) δ 6.2 (d, *J*= 0.4Hz, 1H), 2.6 (t, *J*= 2.3Hz, 2H), 2.3 (t, *J*= 2.5Hz, 2H), 1.6 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.4, 136.2, 114.6, 26.1, 24, 17. GC-MS: *m*/*z* = 112 (M^+) 102, 98, 82, 67, 55.



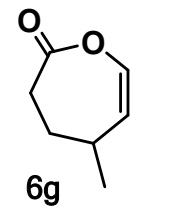
6e

6-methyl-4,5-dihydrooxepin-2(3H)-one (6f): Biotransformation of 100 mg of 3-methyl-cyclohexenone **5f** with 300 mL of expressing $BVMO_{Parvi}$ BL21(DE3)Δ*nem*A cells afforded **6f** (70 mg, 61% yield) as a pale yellow oil. ^1H NMR (300 MHz, $CDCl_3$) δ 6.2 (s, 1H), 2.6 (t, *J*= 3Hz, 2H), 2.3 (m, 2H), 2.1 (m, 2H), 1.7 (s, 3H) ; ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.8, 135, 124, 32.4, 28.2, 25, 18.7. GC-MS: *m*/*z* = 126 (M^+) 110, 98,69,55. HRMS: calcd for $C_7H_{10}O_2$: 127.0754 $[M+H]^+$, found: 127.0752.



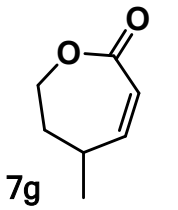
6f

5-methyl-4,5-dihydrooxepin-2(3H)-one (6g): Biotransformation of 100 mg of 4-methyl-cyclohexenone **5g** with 300 mL of expressing $BVMO_{Parvi}$ BL21(DE3)Δ*nem*A cells afforded (*R*)-**6g** (11 mg, 10% yield). ^1H NMR (300 MHz, $CDCl_3$) δ : 6.3 (dd, *J*= 6, 2Hz, 1H), 5.1 (dt, *J*=6, 5Hz, 1H), 2.8 (m, 1H), 2.6 (m, 1H), 2.3 (m, 1H), 2.2 (m, 1H), 1.7 (m, 1H), 1.1 (d, *J*= 7Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ : 172.5, 138.3, 119.7, 33.1, 32.3, 30.1, 20.3. GC-MS: *m*/*z* = 126 (M^+) 112, 96, 81, 71, 67, 55. HRMS: calcd for $C_7H_{10}O_2$: 127.0754 $[M+H]^+$, found: 127.0753.



6g

5-methyl-6,7-dihydrooxepin-2(5H)-one (7g)[18]**:** Biotransformation of 100 mg of 4-methyl-cyclohexenone **5g** with 300mL of expressing $BVMO_{Ocean}$ BL21(DE3)Δ*nem*A cells afforded (*R*)-**7g** (36 mg, 31% yield) as a pale yellow oil. ^1H NMR(300 MHz, $CDCl_3$) δ ; 6.2 (dd, *J*= 13, 4Hz, 1H), 5.9 (dd, *J*= 13, 2Hz, 1H), 4.3 (m, 2H), 2.7 (m, 1H), 2.2 (m, 1H) 1.8 (m, 1H) 1.2 (d, *J*= 7Hz, 3H) ; ^{13}C NMR (75 MHz, $CDCl_3$) δ ; 168.8, 149.2, 119.9, 66.1, 35.7, 34.9, 20.5. GC-MS: *m*/*z* = 126 (M^+) 112, 105, 96, 81, 67, 55. HRMS: calcd for $C_7H_{10}O_2$: 127.0754 $[M+H]^+$, found: 127.0754.



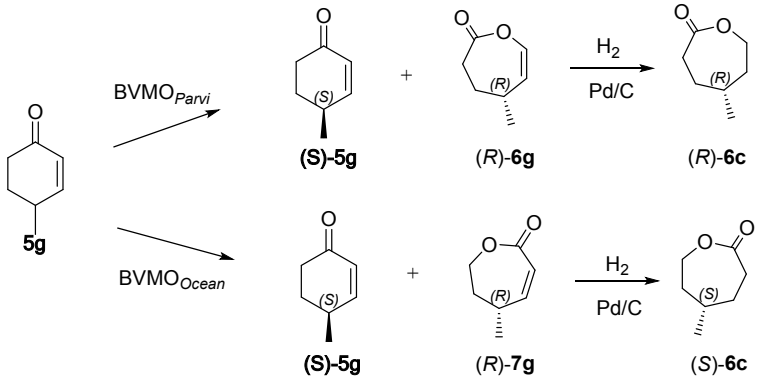
7g

9. Absolute configuration assignments

Absolute configurations of saturated componds were assigned by comparison of enantiomer GC retention times with those of authentic compounds prepared by CHMO mediated biotransformations. Absolute configuration of lactones **6g** and **7g** was determined after reduction into **6c** (H_2, Pd/C) and comparison of enantiomer GC retention times with those of **6c** obtained from CHMO. The chiral columns used are A: Hydrodex γ-DiMOM column (Macherey-Nagel); column B: Chirasil-DEX CB column (Varian).

Table 8: Retention times of the enantiomers of the chiral coumpounds of this study

Ketone	Column	T_{oven} (°C)	t_R Ketone 5 (min)	Column	T_{oven} (°C)	t_R Lactone 6 (min)	t_R Lactone 7 (min)
2-methyl-cyclohexanone **5a**	A	60	9.3 (S) 10.6 (R)	A	110	11.3 (S) 16.5 (R)	-
3-methyl-cyclohexanone **5b**	A	60	10.3 (S) 10.6 (R)	B	100	21.7 (S) 20.6 (R)	18.9 (S) 19.8 (R)
4-methyl-cyclohexanone **5c**	-	-	-	B	100	21.9 (S) 22.6 (R)	-
4-methyl-cyclohexenone **5g**	A	80	6.2 (R) 7.1 (S)	A	130	-	6.2 (R) 6.8 (S)
				A	80	19.4 (R) 20.3 (S)	



Scheme 1. Determination of the absolute configurations of lactones **6g** and **7g**

Determination of absolute configuration of lactones 6g and 7g

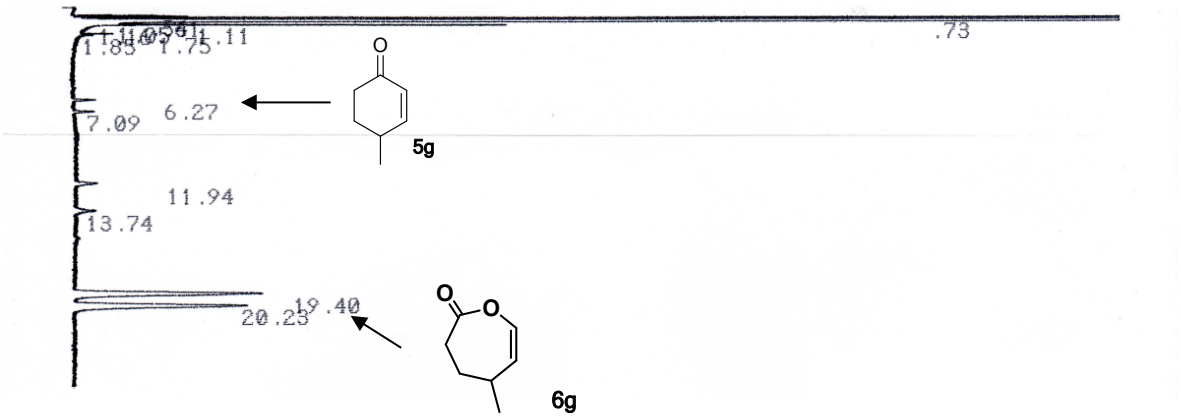


Figure 3: *Rac*- ketone **5g** and lactone **6g** obtained by chemical Baeyer-Villiger oxidation of **5g** and anal yzed on Hydrodex γ-DiMOM column (Macherey-Nagel) at 80°C.

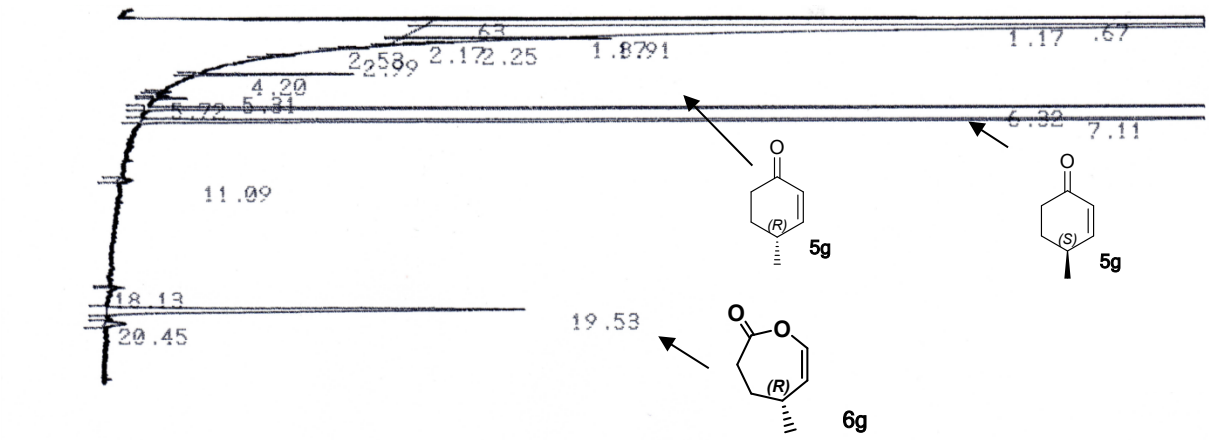


Figure 4 : Biotransformation of **5g**: (S)-Ketone **5g** (ee 30%) and lactone **6g** (ee 93%) produced by $BVMO_{Parvi}$ analyzed on Hydrodex γ-DiMOM column (Macherey-Nagel) at 80°C.

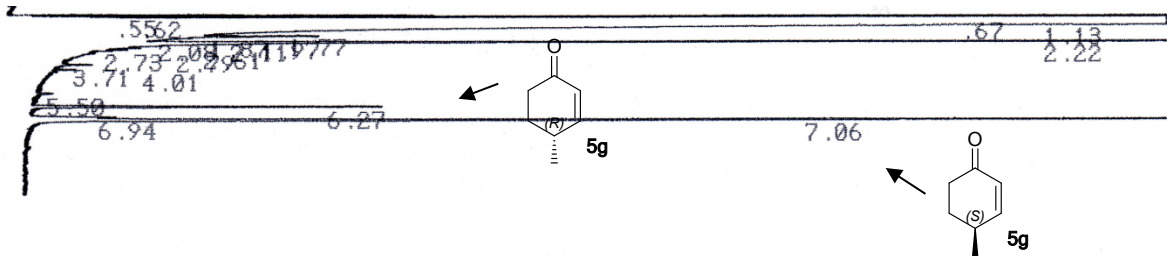


Figure 5: Biotransformation of **5g**: (*S*)-Ketone **5g** (ee 65%) produced by $BVMO_{Ocean}$ analyzed on Hydrodex γ-DiMOM column (Macherey-Nagel) at 80°C.

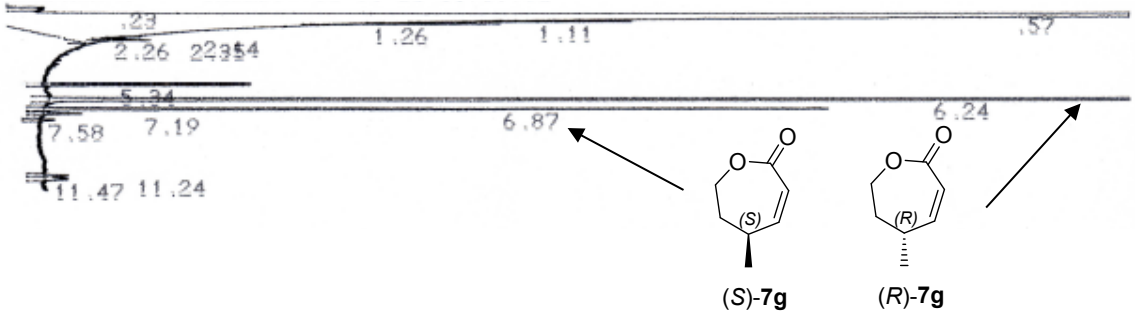


Figure 6 : Biotransformation of **5g**: (*R*)-Lactone **7g** (ee 74%) produced by $BVMO_{Ocean}$ analyzed on Hydrodex γ-DiMOM column (Macherey-Nagel) at 130°C.

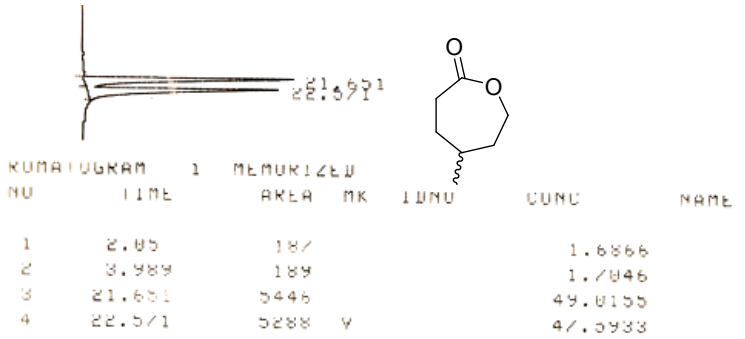


Figure 7: Racemic lactone **6c** obtained from chemical Baeyer-Villiger oxidation of **5c** and analyzed on Chirasil-DEX CB column (Varian) at 100°C.

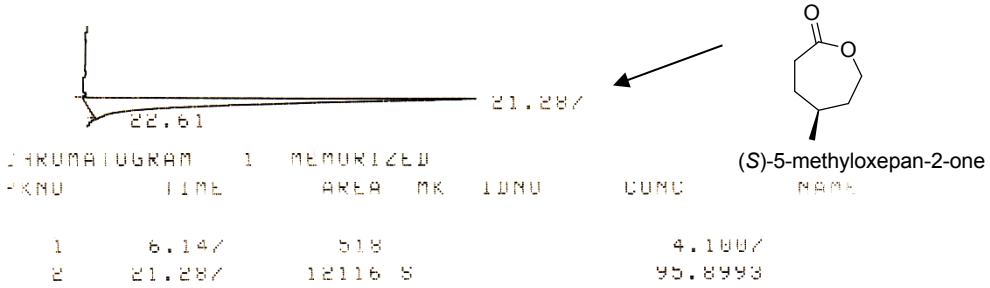


Figure 8: (*S*)-Lactone **6c** obtained from CHMO bioconversion[11,13] of **5c** and analyzed on Chirasil-DEX CB column (Varian) at 100°C.

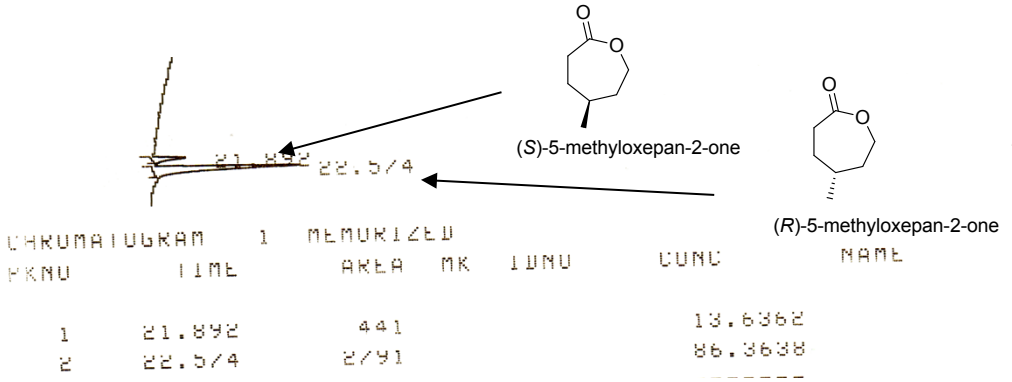


Figure 9: (*R*)-Lactone **6c** obtained after reduction of (*R*)-**6g** produced by BVMO$_{Parvi}$ and analyzed on Chirasil-DEX CB column (Varian) at 100°C.

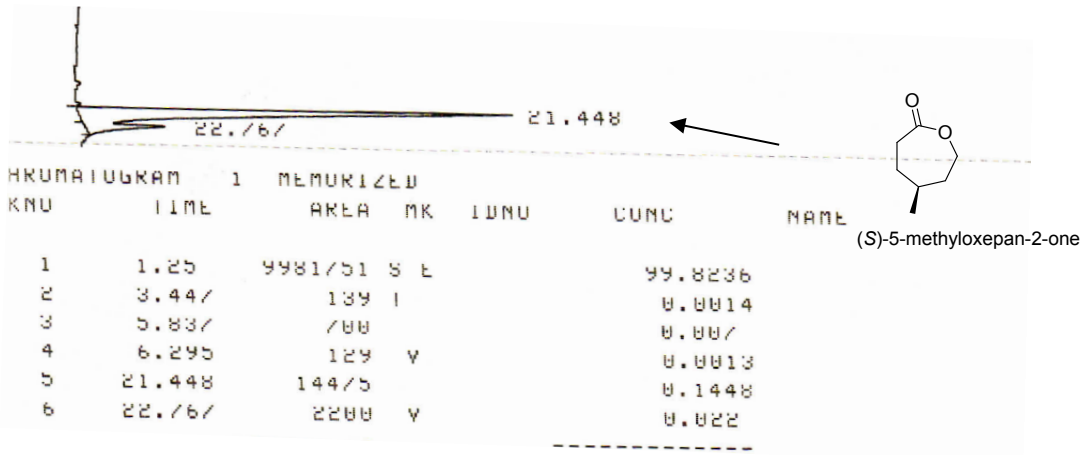


Figure 10: (*S*)-Lactone **6c** obtained after reduction of (*R*)-**7g** produced by BVMO$_{Ocean}$ and analyzed on Chirasil-DEX CB column (Varian) at 100°C.

[1] A. Riebel, H. M. Dudek, G. de Gonzalo, P. Stepniak, L. Rychlewski, M.W. Fraaije, *Appl. Microbiol. Biotechnol.* 2012, **95**, 1479.
[2] M.W. Fraaije, N.M. Kamerbeek, W.J.H. Van Berkel, D.B. Janssen, *FEBS Lett.* 2002, **518**, 43.
[3] K. A. Datsenko, B.L. Wanner, *Proc. Natl. Acad. Sci.* 2000, **97**, 6640.
[4] Baba, T. Ara, M. Hasegawa, Y. Takai, Y. Okumura, M. Baba, K. A. Datsenko, M. Tomita, B. L. Wanner, H. Mori, *Mol. Syst. Biol.* 2006, **2**, 2006.0008. doi:10.1038/msb4100050. Mutant distribution was via GenoBase (http://ecoli.aist-nara.ac.jp/).
[5] C. Aslanidis , P.J. de Jong, *Nucleic Acids Res.,* 1990, **18**, No. 20 6069.
[6] J. H. Miller, *A Short Course in Bacterial Genetics : a Laboratory Manual and Handbook for Escherichia coli and Related Bacteria*. Cold Spring Harbor Laboratory Press, Plainview, NY, 1992.
[7] L. My, B. Rekoske, J. J. Lemke, J. P. Viala, R. L. Gourse, E. Bouveret, *J. Bacteriol.* 2013 **195**, 3784.
[8] B.-D. Chong, Y.-I. Ji, S.-S. Oh, J.-D. Yang, W. Baik, S. Koo. *J. Org. Chem.* 1997, **62**, 9323.
[9] J. D. Stewart, K. W. Reed, J. Zhu, G. Chen, M. M. Kayser, *J. Org. Chem*. 1996, **61**, 7652.
[10] S. Wang, M. M. Kayser,V. J. Jurkauskas, *J . Org. Chem*. 2003, **68**, 6222.
[11] M. D. Mihovilovic, G. Chen, S. Wang, B. Kyte, F. Rochon, M. M. Kayser, J. D. Stewart, *J. Org. Chem.* 2001, **66**, 733.
[12] H. Iwaki, Y. Hasegawa, S. Wang, M. M. Kayser, P. C. K. Lau, *Appl. Environ. Microbiol.* 2002, **68**, 5671.
[13] A. K. Chatterjee, J. P. Morgan, M. Scholl, R. H. Grubbs, *J. Am. Chem. Soc.* 2000, **122**, 3783.
[14] R. M. Moriarty, R. K. Vaid, T. E. Hopkins, B. K. Vaid, O. Prakash, *Tetrahedron Lett*. 1990, **31**, 197.
[15] E. Denmark, N. D. Gould, L. M. Wolf, *J. Org. Chem*. 2011, **76**, 4260.
[16] M. Jiménez-Tenorio, M. C. Puerta, P. Valerga, F. J. Moreno-Dorado, F. M. Guerra, G. M. Massanet, *Chem. Commun.* 2001, **22**, 2324.
[17] R. Aumann, H. Ring, C. Krueger, R. Goddard, *Chem. Ber.* 1979, **112**, 3644.
[18] H. Yanai, A. Takahashi, T. Taguchi, *Tetrahedron* 2007, **63**, 12149.