Pseudomonad Cyclopentadecanone Monoxygenase Displaying an Uncommon Spectrum of Baeyer-Villiger Oxidations of Cyclic Ketones†

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Baeyer-Villiger monoxygenases (BVMOs) are biocatalysts that offer the prospect of high chemo-, regio-, and enantioselectivity in the organic synthesis of lactones or esters from a variety of ketones. In this study, we have cloned, sequenced, and overexpressed in Escherichia coli a new BVMO, cyclopentadecanone monoxygenase (CpdB or CPDMO), originally derived from Pseudomonas sp. strain HI-70. The 601-residue primary structure of CpdB revealed only 29% to 50% sequence identity to those of known BVMOs. A new sequence motif, characterized by a cluster of charged residues, was identified in a subset of BVMO sequences that contain an N-terminal extension of ~60 to 147 amino acids. The 64-kDa CPDMO enzyme was purified to apparent homogeneity, providing a specific activity of 3.94 μmol/min/mg protein and a 20% yield. CPDMO is monomeric and NADPH dependent and contains ~1 mol flavin adenine dinucleotide per mole of protein. A deletion mutant suggested the importance of the N-terminal 54 amino acids to CPDMO activity. In addition, a Ser261Ala substitution in a Rossmann fold motif resulted in an improved stability and increased affinity of the enzyme towards NADPH compared to the wild-type enzyme (Km = 8 μM versus Km = 24 μM). Substrate profiling indicated that CPDMO is unusual among known BVMOs in being able to accommodate and oxidize both large and small ring substrates that include C14 to C15 ketones, methyl-substituted C6 and C7 ketones, and bicyclic ketones, such as decalone and β-tetralone. CPDMO has the highest affinity (Km = 5.8 μM) and the highest catalytic efficiency (kcat/Km ratio of 7.2 × 105 M−1 s−1) toward cyclopentadecanone, hence the Cpd designation. A number of whole-cell biotransformations were carried out, and as a result, CPDMO was found to have an excellent enantioselectivity (E > 200) as well as 99% S-selectivity toward 2-methylcyclohexanone for the production of 7-methyl-2-oxepanone, a potentially valuable chiral building block. Although showing a modest selectivity (E = 5.8), macro lactone formation of 15-hexadecanolide from the kinetic resolution of 2-methylcyclopentadecanone using CPDMO was also demonstrated.

Bacteria of the genus Pseudomonas occupy a visible position in the clinical world and the environment at large. The present taxonomy of Pseudomonas consists of at least 85 validated species (44). Members of this diverse group of aerobic microorganisms have been studied for their medical, environmental, and industrial importance or their relevance in aspects of catabolic plasmids, virulence factors, biofilm formation, solvent tolerance, aromatic metabolism, biodegradation, biotransformations, etc. The many facets of Pseudomonas, including the more recent advancement in comparative genomics, have been captured in an impressive three-volume series (47).

Few Pseudomonas species or strains have been isolated and shown to be capable of metabolizing aliphatic compounds, e.g., linear alkanes (for a review, see reference 63; http://umbbd.ahc.umn.edu/). Even fewer microorganisms have been found to grow on branched or cyclic alkanes (20, 31, 48, 51, 68). Wackett and Hershberger (65) noted that cycloalkanes or alicyclic hydrocarbons represent an “underappreciated” group of molecules, although they are prevalent in nature. Ranging in ring size from 3 to 17 carbons, many cyclic structures are found in steroids, plant oils, fragrances, and a variety of plant secondary metabolites (60). In petroleum mixtures, alicyclic hydrocarbons are estimated to represent up to 12% (wt/wt) of the total hydrocarbons (48). The structure of cyclododecane, a C12 compound, for example, has been compared to the aliphatic bridge components in coal (51). It is possible that activation or functionalization of these cyclic compounds can give rise to value-added products; lactones and dicarboxylic acids useful for polymer synthesis are two prime examples (3, 9, 59).

In this study, we have isolated a Pseudomonas strain that is able to grow on C11 to C15 cyclic ring compounds that include cyclopentadecanol and cyclopentadecanone. In particular, a new flavoprotein Baeyer-Villiger monoxygenase (BVMO), designated cyclopentadecanone monoxygenase (CpdB or CPDMO), was purified and characterized. Substrate profiling indicated it to be an accommodating and versatile biocatalyst in lactone production. In general, the BVMO-mediated ring expansion reaction of cyclic ketone to lactone constitutes a green chemistry alternative to chemical reagents by using molecular oxygen as the oxidant and producing more products than waste, besides offering the prospect of high chemo-, re-

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Acinetobacter sp. strain HI-70 was isolated from a soil sample from Osaka, Japan, by selective enrichment using a minimal salts medium (22), pH 7.6, containing cyclooctadecanol (1.0 g/liter) as the sole carbon source. To the best of available knowledge, this is the first gram-negative organism capable of growth on this large cyclic compound, two previous isolates being gram-positive rhodococci (32, 51). The HI-70 culture was grown on 0.3% succinate as the sole carbon source and 100 μg/ml for HI-70).

Table 1. Bacterial strains and plasmids used in this study

| Bacterial strain or plasmid | Relevant characteristics | Source or reference |
|-----------------------------|--------------------------|---------------------|
| **Bacterial strains** Pseudomonas sp. | | |
| HI-70 | Wild type, grows on cyclooctadecanol or cyclooctadecanone | This study |
| HI-70MB | cpdB::lacZ, Km'; no growth on cyclooctadecanol or cyclooctadecanone | This study |
| E. coli BL21(DE3) | F' ompT hsdSB (rK m- mB) gal dcm | Novagen |
| DH5α | supE44 thi-1 recA1 hsdRI7 endA1 gyrA4 (Nal') Δ(lacI2Z4-argF)U169 deoR [kb80lacΔ(lacZ)M15] | 49 |
| S17-1 | recA pro thi hsdR RP4-2-Tc::Mu-Km::Tn7 Tra- Ty' Sm' | 54 |
| XL1-Blue | recA1 endA1 gryA96 thi hsdR17 supE44 relA1 [F' lacP'ZM15 Tn10(Tet')] | 49 |
| **Plasmids** | | |
| pARO190 | Mobilizable vector; Ap' | 45 |
| pARO-cpdb | pARO190 with a 2.9-kb EcoRV fragment containing cpdB from pCD200 | This study |
| pARO-cpdb::lacZ | cpdB::lacZ-Km'; Sall-excised lacZ-Km' cassette from pKOK6.1 in Xhol site of pARO-cpdb | This study |
| pKOK6.1 | lacZ-Km' cassette | 30 |
| pSD80 | Expression vector with tac promoter; Ap' | 55 |
| pUC19 | Cloning vector with lac promoter; Ap' | 71 |
| pCD200 | pUC19 with a 4.2-kb BclI fragment from Pseudomonas sp. strain HI-70; Ap' | This study |
| pCD201 | 1.8-kb EcoRI' fragment containing cpdB in pSD80; Ap' | This study |
| pCpdbBdelN | Deletion of CpdB N-terminal 54 amino acids in pCD201 | This study |
| pCpdbBdel7 | Deletion of internal 7 amino acids in CpdB in pCD201 | This study |
| pCpdbBS261A | Replacement of Ser261 by Ala in pCD201 | This study |
| pCpdbBG242A | Replacement of Gly242 by Ala in pCD201 | This study |

* EcoRI restriction endonuclease site introduced by PCR design.

**MATERIALS AND METHODS**

Bacterial strains, culture conditions, and plasmids. All bacterial strains and plasmids used in this study are listed in Table 1. Pseudomonas sp. strain HI-70 was isolated from a soil sample from Osaka, Japan, by selective enrichment using a minimal salts medium (22), pH 7.6, containing cyclooctadecanol (1.0 g/liter) as the sole carbon source. To the best of available knowledge, this is the first gram-negative organism capable of growth on this large cyclic compound, two previous isolates being gram-positive rhodococci (32, 51). The HI-70 culture was grown on 0.3% succinate as the sole carbon source and 100 μg/ml for HI-70).

Sequencing of the 16S rRNA. The near-full-length 16S rRNA gene of strain HI-70 was amplified by PCR as previously described (23). Genomic DNA was obtained by the method of Wilson (70). The DNA sequencing reaction was carried out using the ABI PRISM BigDye Terminator (v. 3.1) cycle-sequencing kit (Applied Biosystems) in a GeneAmp PCR system 9700 (Perkin-Elmer). The thermal profile included 25 cycles of 30 s at 96°C (denaturation), 5 s at 50°C (annealing), and 4 min at 60°C (DNA extension). The sequencing products were purified using a CENTRISEP column (Princeton Separations) and analyzed in an automated fluorescence sequencing model (373A; Applied Biosystems). 16S rRNAs for comparison were obtained from the GenBank sequence database of the National Center for Biotechnology Information (NCBI; Bethesda, MD).

Cloning of the CPMO-encoding (cdpB) gene. Two conserved amino acid regions of the cyclohexane monoxygenase (CHMO) (22), cyclooctanone monoxygenase (CMO) (23) and steroid monoxygenase (42) were chosen to design two degenerate primers (5'-GGGAATTCGGATCCTCAATAACCAGGCT3'- and 5'-GCATCTGCAGGTGCTGCTGCGCTGCTG-3') for PCR amplification (1 = inosine; N = T, C, A, or G; R = A or G; y = C or T). The nucleotides correspond to amino acid positions 46 to 52 and 377 to 382, respectively, of the corrected Acinetobacter sp. strain NCIMB CHMO sequence (22, 29). The amplified DNA fragment (~1 kb) was labeled by the digoxigenin-11-UTP system according to the manufacturer’s instructions (Roche Molecular Biochemicals) and used to probe a Southern hybridization of strain HI-70 genomic DNA digested with a number of restriction enzymes. As a result, a 4.2-kb BclI fragment that was probed positive was chosen for cloning. This DNA was subsequently isolated from a 0.8% agarose gel and ligated to the E. coli plasmid pUC19, which had been linearized with BcI and dephosphorylated (49). Colony hybridization using the PCR product as a probe was carried out to screen for a positive clone. The resulting recombinant plasmid transformed in E. coli XL1-Blue was designated pCD200.

Inactivation of cdpB in strain HI-70. The cdpB disruption mutant was constructed by inserting a lacZ-Km' cassette from pKOK6.1 in Xhol site of pARO-cpdb. The lacZ-Km' cassette was excised as a Sall fragment and inserted into the Xhol site within the cdpB gene in pARO-cpdb, yielding pARO-cpdb::lacZ. The plasmid was inserted into strain HI-70 from E. coli S17-1 (pARO-cpdb::lacZ) by transconjugation. Km' colonies were selected on minimal salts medium plates containing 0.3% succinate as the sole carbon source and 100 μg/ml kanamycin. Ap' colonies were selected from Km' colonies as a double crossover mutant and used for further experiments.

Subcloning of the cdpB gene in E. coli BL21. Two primers containing an EcoRI restriction site (underlined) were synthesized (5'-CGGAATTCATAGATGCACT AATTCAGAGCC-3' and 5'-CGGAATTCATACGCTGCGCTGCTG-3') and used to amplify the cdpB gene contained in a 1.8-kb DNA fragment from pCD200. Pfu polymerase was used for the PCR amplification, and the thermal profile included 30 cycles of 1 min at 94°C, 1 min at 50°C, and 3 min at 72°C. The target DNA fragment was separated by gel electrophoresis on 1% agarose (1× Tris-acetate-EDTA), excised, and purified by the QIAEX II gel extraction kit (QIAGEN). The purified DNA and the pSD80 vector were each digested with EcoRI, ligated, and transformed into E. coli BL21 cells. The recombinant plasmid is designated pCD201. DNA sequencing was performed to exclude possible mutations of the amplified cdpB gene.

Growth conditions and cell disruption of E. coli BL21(pCD201). E. coli BL21 harboring the pCD201 plasmid was maintained on LB medium containing glyc erol (50%, vol/vol) at ~80°C. For protein expression experiments, a fresh LB agar plate (1.5% agar) containing ampicillin (50 μg/ml) was prepared from the stock culture, and one colony was transferred to a 10-ml preculture (LB medium containing ampicillin, 50 μg/ml) and grown overnight. Main cultures were carried out in 2-liter Erlenmeyer flasks containing 1 liter LB medium and am picillin (50 mg), using 3 ml of the preculture. Cells were grown at 30°C, and CdpB production was induced by the addition of IPTG (isopropyl-β-D-thiogalactopyranoside) (1 mM). About 3 h after induction, the cells were harvested by cen-
trifugation (5,000 × g, 20 min, 4°C) and washed twice with 20 mM Tris-HCl (pH 7.2). The pellet was resuspended in the same buffer, and crude extract was obtained by passing the cell suspension twice through a French pressure cell (SLM Instruments, Urbana, Ill.) at 20,000 lb/in2 followed by centrifugation (20,000 × g, 30 min, 4°C) to remove broken cells and debris.

**Enzyme assay and kinetics.** The CPDMO enzyme activity was assayed in a reaction mixture (1 ml) containing Tris-HCl buffer (50 mM, pH 9.0), 0.1 mM NADPH, and an appropriate amount of enzyme (10 to 20 μM), and the reaction was started by adding 10 μl of substrate (e.g., 0.1 M cyclopentadecanone in n-propanol). The decrease in the absorbance of NADPH was measured spectrophotometrically at 340 nm. The specific activity was defined as μmol of NADPH (ε = 6.3 liter mmol⁻¹ cm⁻¹) oxidized per minute (U) per milligram of protein (U/mg).

Protein purification. All purification procedures were performed at 4°C on an LKB II fast protein liquid chromatography system (Pharmacia, Uppsala, Sweden). The crude extract (one 10-ml aliquot per run, of a total of 45 ml) was boiled in sodium phosphate buffer, 50 mM, pH 7.5) was boiled to denature it (Table 2). The crude extract (one 10-ml aliquot per run, of a total of 45 ml from a 4-liter cell culture) was loaded on a Mono Q column (10/100) previously equilibrated with 20 mM Tris-HCl buffer (pH 7.2) (buffer A) by using a 10-μl superloop. The flow rate was 0.75 ml/min. The column was washed with buffer A until no protein could be detected in the flowthrough, and the enzyme was subsequently eluted with a linear gradient of 0 to 0.2 M NaCl in buffer A. Active fractions were collected, pooled, and concentrated by ultrafiltration (membrane exclusion size, 30 kDa) (in a 50-ml stirring cell; Amicon, United States) and applied to a Superose 6 HR (10/300) gel filtration column previously equilibrated with 20 mM Tris-HCl buffer (pH 7.2) containing 150 mM NaCl (buffer B). Protein was eluted with the same buffer (flow rate of 1 ml/min) and collected in 0.5-ml fractions. Active fractions were pooled and concentrated by ultrafiltration and applied to a HiPrep Sepharoc S-200 column (26/60) which had been previously equilibrated with buffer B. Protein was eluted using buffer B (flow rate of 2 ml/min), and active fractions were collected, pooled, and concentrated by ultrafiltration. The protein profile was monitored by its absorbance at 280 nm.

**TABLE 2. Purification of cyclopentadecanone monoxygenase**

| Purification step | Total protein (mg) | Total activity (U) | Sp act (U/mg) | Yield (%) | Purification (fold) |
|-------------------|-------------------|-------------------|--------------|-----------|-------------------|
| Crude extract     | 1,332             | 373               | 0.28         | 100       | 1                 |
| Mono Q            | 117               | 214               | 1.83         | 57        | 6.5               |
| Superose 6        | 62                | 156               | 2.52         | 42        | 9                 |
| Sephacyr S200     | 19                | 75                | 3.94         | 20        | 20                |

After the Superose 6 step, the protein was concentrated and dialyzed against buffer B, and active fractions were collected, pooled, and concentrated by ultrafiltration (membrane exclusion size, 30 kDa) (in a 50-ml stirring cell; Amicon, United States) and applied to the C8 column previously equilibrated with buffer B. Protein was eluted with the same buffer (flow rate of 1 ml/min) and collected in 0.5-ml fractions. Active fractions were pooled and concentrated by ultrafiltration and applied to an HPLC system (Waters 600E liquid chromatography system (Waters 600E solvent delivery system, autosampler model 717, PDA detector model 996) on a reversed-phase column (CSC-Incertis, 150A/ODS2, 5 μm, 25 cm by 0.46 cm; CSC, Montreal, Canada). The mobile phase consisted of 25% methanol in sodium phosphate buffer (10 mM, pH 6.0), and the flow rate was 1 ml/min (isocratic) at a constant temperature of 45°C. Different retention times of flavin adenine dinucleotide (FAD) (5.8 min) and flavin mononucleotide (FMN) (7.8 min) using pure standards (Sigma) were recorded (at 450 nm) and compared with the retention times of the sample.

**Truncation and mutagenesis of cpdB sequence.** Four mutations in the CpdB sequence were constructed. Plasmid pCpdb-delN, lacking the first 54 amino acids of the N terminus of CpdB, was created as follows: primer Cpdb-delN (GGGATTCATGAAGACCCCTATGGCGGA) containing an EcoRI restriction site (underlined) and ATG start codon (double underlined), was designed to amplify the truncated cpdB gene together with a C-terminal primer that was used for the subcloning of the cpdB gene. Pfu polymerase was used for the PCR amplification, and the thermal profile included 30 cycles of 1 min at 94°C, 1 min at 50°C, and 3 min at 72°C. The target DNA fragment was separated by gel electrophoresis on 1% agarose (1× Tris-acetate-EDTA), excised, and purified by the QIAEX II gel extraction kit (QIAGEN). The purified DNA and the pSD80 vector were each digested with EcoRI, ligated, and transformed in E. coli BL21 cells. The recombinant plasmid was designated pCpdb-del. DNA sequencing was performed to exclude possible mutations of the amplified cpdB gene.

A second mutant (pCpdb-delT) with a deletion of seven amino acids at positions 240-QTGNLEG-246 was carried out using the site-directed, ligase-independent mutagenesis procedure of Chiu et al. (10) as follows: four primers (two totally and two nonpaired), per Chiu et al. terminology, were synthesized to facilitate the desired deletion. In the primer design, the position of the deleted DNA sequence defines the placement of the other regions. The 5′- adaptor tail (18 bases, underlined), the region that will make up the overlap of the tail primers [CDNB R1, 5′-AACGCGCCCGTGAATGATCGTACCGGACATTGTTGGT (GGCGGC)] must be at least 4 nt and immediately 5′ to the FR primer tail. CDNB Fn, 5′-TTGAAGAAGCAACCGCGGTCC (GGCGGC), is the complementary oligonucleotide region that is recognized by the gene-specific portion of the primers (normal capital letters) immediately 5′ to the 5′ adaptor tail. CDNB Rn, 5′-(TGGATCCTACCGGACATTGTTGGT), is the 3′ gene-specific region (in bold) immediately 3′ to the deleted sequence. Sequence deletion from pCpdb is accomplished by reducing the desired deletion region from the single PCR amplification described as follows.

A single PCR was performed containing the following components: 2.5 μl of 10× Pfu buffer, 200 μM each deoxynucleoside triphosphate, 1 mM MgSO4, 100 mM betaine, 10 pmol of each of the four primers, 10 pg of the 7.676-kb plasmid template pCD201, 0.5 U Taq DNA polymerase (New England Biolabs, Beverly, MA), 0.25 μl Platinum Pfu DNA polymerase (Invitrogen, Carlsbad, CA), and molecular biology-grade water to a final volume of 25 μl. The reaction was started by heating to 98°C for 2 min and then brought to 85°C at which point the DNA polymerase mix was added. The PCR was subjected to a further 25 cycles of 95°C for 15 s, 58°C for 20 s, and 68°C for 3.5 min, adopting the conditions of the site-directed, ligase-independent mutagenesis technique. The PCR mixture was amplified on a 0.8% agarose gel, and the 7.676-kb fragment was isolated and purified with QIAEX II. An aliquot (100 to 200 μg) of linearized pCpdb-del7 was cloned into pBluescript II (Stratagene) in an H buffer (300 mM NaCl, 50 mM Tris [pH 9.0], 20 mM EDTA [pH 8.0]) and used for heteroduplex formation by denaturation at 99°C for 3 min followed by two hybridization cycles: 65°C for 5 min and 30°C for 15 min. One-third of the heteroduplex reaction mixture was used to transform CaCl2-competent E. coli BL21(DE3) cells.

Clones were screened by PCR for the desired deletion using detection primers DEL R, 5′-(CGAGCCCTTTCGCCGCTGGCCTGCTC); corresponding to the residues 414-ERITEKGL-421 of the CpdB sequence, and DEL F, 5′-(GGGCGCGAATTGAAAAAGACA) corresponding to the new sequence created by the seven-amino-acid deletion. Colonies were picked and grown, and plasmid DNA template was prepared using the QIAprep spin miniprep kit (QIAGEN, Hilden, Germany). An aliquot of 1 μl of plasmid DNA was used as template in a PCR (screening) mixture that contained 2.5 μl of 10× Taq buffer, 200 μM of each deoxynucleoside triphosphate, 10 pmol each of the DEL F and DEL R primers, 1 U of Taq DNA polymerase, and molecular biology-grade water to a final volume of 25 μl. Positive clones were identified by electrophoresis on a 0.8% agarose gel for the presence of a 547-bp fragment that would confirm the desired deletion. Further confirmation of the desired deletion was carried out by DNA sequencing.

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RESULTS

16S rRNA identification and growth substrates of strain HI-70. The 1,475-base-long 16S sequence of strain HI-70 was found to be 99% identical to that of Pseudomonas pseudocaligines strain KF707, a well-known biphenyl/polychlorinated biphenyl degrader (18); high percentages of identity were also found with Pseudomonas resinovorans strain ATCC 14235T (98%), Pseudomonas stutzeri strains SP1402 and LS401 (97%), and several other pseudomonads not identified to the species level (BLAST search results not shown). The HI-70 strain is tentatively referred to as a Pseudomonas sp. Besides its growth on cyclodecane and cyclocodcanone as a sole carbon and energy source, strain HI-70 is also able to grow on cycloundecane (C11), cyclooctadecane (C18), cyclopentadecanone (C15), and cyclopentadecanone (C15). It did not grow on smaller cyclic C6 to C10 compounds, such as cyclopentanol, cyclopentanone, cyclohexanol, cyclohexanone, cycloheptanol, cycloheptanone, cyclooctanol, cyclooctanone, cyclononanone, and cyclocodcanone. Methyl-substituted compounds, e.g., cis/
trans 2-methyl cyclohexanol and 2-methyl cyclohexanone, did not serve as growth substrates either.

Cloning and sequencing of a 4.2-kb BclI fragment containing cpdB. The primers that were designed from two conserved regions of several known BVO sequences amplified the expected ~1-kb DNA fragment. A partial sequence was determined prior to using it as a probe to clone a 4.2-kb BclI genomic fragment of strain HI-70 in an *E. coli* pUC19 vector to produce the recombinant plasmid, pCD200. DNA sequencing of the insert (4.216 kb) and computer analysis revealed the presence of a 601-codon open reading frame that we designated *cpdB* (described in more detail below). This is flanked by genes for a LysR-type transcriptional regulator (designated *cpdR*) upstream in the opposite orientation and a possible lactone hydrolase (*cpdC*) downstream in the same orientation as *cpdB* (Fig. 1). The respective intergenic spaces are 409 bp and 94 bp, each containing the appropriate Shine-Dalgarno sequences; a possible promoter element (TTGACA---GAGAAT) is readily identified in the 409-bp intergenic sequence immediately before the *cpdB* gene but not in the *cpdB*-*cpdC* intergenic space. The 304-amino-acid CpdR is most related to a “probable transcriptional regulator” (GenBank no. AE004683.1) in the *Pseudomonas aeruginosa* PAO1 genome showing 34% sequence identity. CpdC was found to be a new member of the large family of lipase/esterases; the closest homologous sequence identity. CpdC was found to be a new member of the large family of lipase/esterases; the closest homologous sequence identity (GenBank no. AAS77233.1) was described in more detail below. This is flanked by genes for a LysR-type transcriptional regulator (designated *cpdR*) upstream in the opposite orientation and a possible lactone hydrolase (*cpdC*) downstream in the same orientation as *cpdB* (Fig. 1). The respective intergenic spaces are 409 bp and 94 bp, each containing the appropriate Shine-Dalgarno sequences; a possible promoter element (TTGACA---GAGAAT) is readily identified in the 409-bp intergenic sequence immediately before the *cpdB* gene but not in the *cpdB*-*cpdC* intergenic space. The 304-amino-acid CpdR is most related to a “probable transcriptional regulator” (GenBank no. AE004683.1) in the *Pseudomonas aeruginosa* PAO1 genome showing 34% sequence identity. CpdC was found to be a new member of the large family of lipase/esterases; the closest homologous sequence identity (GenBank no. AAS77233.1) was derived from an uncultured organism.

Inactivation of the *cpdB* gene. The *cpdB* gene was chromosomally inactivated by transcriptional fusion of *cpdB*:lacZ, using a lacZ-Km' cassette from the pKOK6.1 vector (30). The resulting mutant, *Pseudomonas* sp. strain HI-70MB, was unable to grow on cyclo Dodanone, cyclotridecanone, cyclopentadecanol, and cyclopenta decanone, which are otherwise carbon and energy sources for strain HI-70. In LB medium, a low level (<20 Miller units) of β-galactosidase expression was observed in HI-70MB cells. The presence of cyclo Dodanone or cycloDodanone increased the activity to 2,128 or 1,089 Miller units, respectively, after 15 h of cultivation. This observation indicated a possible regulation of transcription of *cpdB* gene by *cpdR* that is located upstream. However, regulation is outside the scope of this study.

Sequence characteristics of CpdB. The conserved stretches of amino acids in CpdB, typical of BVO sequences, are those of two possible Rossmann folds (79-GGGFGG-84 and 256-GTQATS-261), a so-called BVO fingerprint (221-FKGHSHFH

TSRWD-232), and two other motifs (109-GG-110 and 431-DCLIYATG-438) (15, 62, 69). The underlined sequences correspond to the invariant or most-conserved residues in the various aligned BVO sequences (15). The three highest scores in a BLAST search showing 49% to 54% sequence identity were provided by putative monooxygenases derived from the genome sequences of *Bradyrhizobium japonicum* strain USDA 110 (btr3857; gi 47118316), *Streptomycyes avermiti lis* strain MA-4680 (gi 57546753), and *Mycobacterium avium* subsp. *paratuberculosis* (gi 41398205), followed by a known BVO (cyclo Dodanone monooxygenase, CddA; CDMO) that originated from *Rhodococcus ruber* strain SCI (32). In a phylogenetic analysis (result not shown), CpdB is in a distinct cluster from the majority of the cloned BVOs that function with lower cyclic ring compounds, steroids, or benzene ring-based substrates. Examples of the latter are the classical CHMO of *Acinetobacter sp.* sp. (8, 22, 29), CPMO of *Comamonas* sp. strain NCIMB 9872 (23), steroid monooxygenase (42), hydroxyacetophenone monooxygenase (HAPMO) (25), and phenylacetone monooxygenase (PAMO) (17, 37).

N-terminal amino acid sequencing confirmed the first 10 residues (SQIIQEPAAEA) of the CpdB protein predicted by its DNA sequence, while also establishing the fact that the CpdB protein is characterized by an N-terminal extension of 66 amino acids compared to the prototype CHMO sequence (Fig. 2A). This is like the CDMO or CddA of *R. ruber* SCI and the HAPMO of *Pseudomonas fluorescens* strain ACB (25). Interestingly, taken together with four other uncharacterized BVO sequences in the GenBank microbial database, it appears that a subset of BVO sequences arises that is characterized by having an N-terminal extension ranging from 60 to 147 amino acids (numbered from the first G of the first consensus Rossmann fold sequence). A particularly interesting feature within this extension is a stretch of about 20 amino acids that is predominantly charged (consensus DLxALRxKxRYRxERDKRLRx XD).

Another notable feature of the CpdB sequence is an apparent insertion of 10 or 11 amino acids (amino acids 240 to 249) in between the “BVO fingerprint” (amino acids 221 to 232) and the second Rossmann fold sequence (amino acids 256 to 261; Fig. 2B). This is also found in CDMO and the four uncharacterized BVO sequences (Fig. 2B).

Biochemical properties and purification of CpdB. Knowledge of the *cpdB* nucleotide sequence was used to construct an overexpression clone in the IPTG-inducible plasmid pSD80 in order to verify its protein production and enzyme activity and
to facilitate protein purification. In pCD201, the CpdB protein was found to be expressed readily, with an observed molecular mass of 63.5 kDa by SDS-PAGE analysis which confirms the predicted molecular mass of 68,313. No appropriate protein band of this size was detectable in the control cells containing the pSD80 vector only. By gel filtration, the molecular mass of the native CdnB was found to be 64 kDa, indicating a monomeric enzyme.

The enzyme was purified to homogeneity using a three-step procedure, based on anion exchange chromatography on Mono Q and two steps of molecular size exclusion chromatography on Superose 6 and Sephacryl S200, respectively. The specific activity of CpdB was enriched about 14 times, rendering 3.94 U/mg and a yield of 20% (Table 2). Several other purification trials using ammonium sulfate precipitation followed by hydrophobic interaction chromatography (phenyl, butyl, and octyl Sepharose) failed to produce an active enzyme preparation.

**Cofactor and stability of CpdB.** The free FAD concentration was determined by HPLC using the supernatant of boiled purified enzyme. This was estimated to be 0.65 mol/mol protein. However, in the same fraction, 0.11 mol/mol FMN was detected (data not shown). CpdB requires NADPH for its activity; replacement by NADH resulted in greatly decreased catalytic activity (<5%). The Km for NADPH was established to be 24 μM.

In crude extracts, CpdB has a half-life of about 50 days at 4°C, whereas the purified enzyme retained its full activity when stored at −80°C for several months. Occasionally, protein precipitation was observed when CpdB was stored at 4°C for longer terms (>1 week), which could be prevented in part by adding FAD (protein:FAD ratio, 1:10). However, enzyme ac-
tivity loss could not be recovered by the addition of FAD or FMN. Furthermore, heating of purified CpdB during the denaturing step in SDS sample buffer using reducing conditions (>5 min at 95°C) led to total degradation of the protein accompanied by the appearance of many additional bands in SDS-PAGE (data not shown).

Substrate specificity and kinetic properties of CpdB. Optimum enzyme activity was obtained at 40°C and pH 9.0 using Tris-HCl buffer (50 mM). CpdB has the highest affinity towards cyclopentadecanone (5.8 μM) and the highest reaction speed (k_{cat}) towards cyclotridecanone and methylcyclopentadecanone (6.5 and 6.6 s⁻¹, respectively). However, the catalytic efficiency (k_{cat}/K_m) favors cyclopentadecanone (7.2 × 10⁵ M⁻¹ s⁻¹) as a substrate (Fig. 3).

CpdB is also able to oxidize a variety of alkyl-substituted cyclic ketones, particularly 2-methyl-, 2-methoxy-, and 2-ethoxycyclohexanone (Fig. 4). The activity of CpdB towards these substrates is 29 to 34% higher than that of cyclopentadecanone. However, the respective K_m values are 790, 80, and 170 μM, compared to 6 μM for cyclopentadecanone. 4-Ethylcyclohexanone, in contrast to 4-methylcyclohexanone or 3-methylcyclohexanone, appeared to be a good substrate, having 80% of the cyclopentadecanone activity.
activity, but the affinity is low. Interestingly, the dimethyl-substituted 2,6-dimethylcyclohexanone is a poor substrate compared to the 2-methylated ketone.

CpdB is also active towards saturated bicyclic ketones, such as 1- and 2-decalone. Both of these compounds displayed an equivalent high catalytic activity, but the affinity appears to favor 1-decalone with a $K_m$ value of 21 $\mu$M that approximates those of the large cyclic compounds. CpdB is also capable of using tetralones as substrates, but the enzyme appears to have a preference for substitutions of the keto group in the 2- or $\beta$-position, viz., 1-methyl-2-tetralone and $\beta$-tetralone. The results confirmed that conjugated ketones were generally poor substrates for this enzyme. Chloro-substituted cyclic ketones, such as 2-chloro-cycloheptanone, 2-chloro-cyclooctanone, and 2-chlorocyclopentadecanone, are poor substrates for CpdB (results not shown).

Reactor detection of product formation. Using purified CpdB and cyclooctanone as the substrate, we monitored product formation (lauryle lactone) and substrate depletion with Fourier transform IR spectroscopy-based ReactIR 4000 spectroscopy. Figure 5 shows the overlay spectra obtained for the conversion of cyclooctanone (1,714 cm$^{-1}$) to lauryle lactone (1,741 cm$^{-1}$) as a function of time. The oxidations of cyclopentadecanone and 2-methylyclopentadecanone were also followed using the React IR4000, and their lactone products were confirmed by extraction with ethyl acetate (EtOAc) and identified by comparison with authentic samples on GC-MS and capillary GC (data not shown).

Truncation and mutagenesis of CpdB. (i) pCpdB-delN. To probe the possible function of the CpdB N-terminal extension, pCpdB-delN was created, which lacked the first 55 codons of cpdB. As a result, the mutant protein with the correct molecular size (62 kDa; calculated size, 61,876 Da) was found to be expressed as a soluble protein. N-terminal sequencing confirmed the first 10 amino acids of the truncated CdnB protein (MKDPYADP4V; methionine was introduced by the PCR primer). However, this truncated protein had less than 5% of the wild-type activity.

(ii) pCpdB-del7. Deletion of amino acids 240-QTGNL246 in pCpdB-del7 produced a correctly sized protein that was found in the particulate fraction and enzymatically inactive (data not shown). No attempt was made to solubilize the inclusion bodies.

(iii) G242A and S261A mutants. In contrast to pCpdBdel7, a substitution within the seven-amino-acid region (G242A mutant) and a substitution within the second Rossmann fold motif (S261A mutant) produced a soluble and active protein of the equivalent quantity as the native protein. Kinetic parameters of the purified enzymes from these mutants were determined and compared to the wild-type enzyme (Table 3). In general, the catalytic efficiency ($k_{cat}$/K$_m$) of the G242A mutant did not change significantly for the three substrates and NADPH. In contrast, the S261A mutant showed an apparent increase in affinity towards NADPH as indicated by a threefold-lower $K_m$ than that of the wild-type protein (Table 3). The S261A mutant also showed an increased stability (75% activity after 50 days at 4°C) compared to the wild-type enzyme, whereas the G242A mutant showed a decreased activity and stability (half-life of about 30 days at 4°C; results not shown).

Kinetic resolution of 2-methylyclopentadecanone (compound no. 1). (The compound numbers refer to the bold and underlined numbers in Tables 4 and 5). Whole-cell biotransformation was carried out using E. coli BL21(DE3)(pCD201) cells induced by IPTG to test the possibility of production of enantiopure 15-hexadecanolide as a possible application of CPDMO in asymmetric organic synthesis (33). It turned out that only 10%...
conversion could be achieved with one-stage fermentation, and the lactone (compound no. 2) had 65% ee \((S)\). A prolonged fermentation (three stage) yielded 30% conversion and a lactone ee of 59% (Table 4). The biotransformation mixture was extracted by EtOAc and monitored using two chiral-phase GC columns. The Supelco Beta DEX 225 chiral-phase column did not resolve the lactone enantiomers, but it could separate the ketone substrate and lactone product. This problem was resolved by using a Chrompak Chiralsil-Dex CB column as previously described (36). The absolute configuration of 15-hexadecanolide was also assigned by comparison with the value in the literature (36).

**Biotransformation products of small monocyclic ketones.** In whole-cell biotransformation, 2-, 3-, or 4-methyl-substituted cyclohexanones all gave good percent conversions. In particular, 2-methylcyclohexanone (compound no. 3) gave an excellent enantioselectivity, \(E > 200\). CPDMO oxidized it with complete \((S)\)-selectivity, giving a product (compound no. 4) with specific rotation \([\alpha]_D\) of \(-16, c 10, \text{in CH}_2\text{Cl}_2\) and >99% ee in chiral-phase GC determination.
hexanone (compound no. 5) gave two regioisomers of lactone, compound no. 6b and compound no. 6a (the ratio of lactone was 1:3), although with high enantioselectivity. Oxidation of 4-methyl and 4-ethyl cyclohexanones showed that CPDMO behaved like the CHMO of *Acinetobacter* sp. strain NCIMB 9871 that gave the highly optical pure lactone by desymmetrization of prochiral substrate (57).

4-tert-Butyl cyclohexanone (compound no. 11) was trans-

| Substrate | Conversion (%)<sup>a</sup> at 20 hrs | Enantioselectivity<sup>d</sup> | Product ee<sup>b</sup> (conv. yield) |
|-----------|-------------------------------|-----------------|---------------------------------|
| 4-tert-butyl cyclohexanone | 68% | 99% ee (S)<sup>d</sup> | 99% ee (S)<sup>d</sup> (72%<sup>f</sup>) at 28 hrs |
| 2,6-dimethyl cyclohexanone | 74% | 99% ee (?)<sup>d</sup> | 99% ee (80%) |
| 2-methylcyclopentanone | 10% | E = 15<sup>e</sup> | 87% ee (10%) |
| 3-methylcyclopentanone | <3% | E ~ 1 | 0% ee (<3%) |
| 2,4,4-trimethyl cyclohexanone | 78% | E = 15<sup>e</sup> | 41% ee (82%) at 24 hrs |

<sup>a</sup> Conversion yield was based on the EtOAc-extracted sample determined by chiral-phase GC analysis on a β-Dex 225 column or Chrompak Chiral-Dex CB column and referred to an internal standard.
<sup>b</sup> ee, enantiomeric excess equal to [R] – [S]/[R] + [S] (%). Values of ee were based on chiral-phase GC analysis. conv. yield, conversion yield.
<sup>c</sup> E(ce), E(enantioselective ratio) value calculated based on product ee(P) and remaining substrate ee(S). E = \(\ln\left(\frac{1 - ce(S)}{1 + ce(S)/ce(P)}\right)\). (46) or based on product ee(P) and conversion yield referred to in equation in footnote e.
<sup>d</sup> (S) or (R), absolute configuration of products.
<sup>e</sup> E(ce(P)), E(enantioselective ratio) value calculated based on product ee(P) and conversion yield e, then E(ce(P)) = \(\ln\left(1 - ee(S)/(1 + ee(S)/ee(P))\right)\). (7).
<sup>f</sup> Isomer ratio is the ratio of two regioisomeric lactones.
<sup>g</sup> Enantioselectivity is the product ee for prochiral substrate and E (enantioselective ratio) for racemate substrate.
<sup>h</sup> Enantioselectivity (E) calculated from the plot at 4 h, 32% conversion; the regioisomer ratio was 1 (945% ee):5.2 (99% ee).
formed to the corresponding lactone (compound no. 12) in good yield and excellent enantioselectivity of 99% ee (S). CPDMO also accepts cis-2,6-dimethylcyclohexanone (compound no. 13) as a substrate, with 74% conversion yield at 20 h with high enantioselectivity (99% ee). Since the 2,6-dimethylcyclohexanone sample was a cis/trans mixture (5% trans-isomer), we also found that trans-2,6-dimethylcyclohexanone was a relatively poor substrate of CPDMO.

The five-member ring ketone system generally gave a poor performance (Table 4). However, a multi-methyl-substituted cyclopentanone such as 2,4,4-trimethylcyclopentanone (compound no. 19) turned out to be a good substrate; it was oxidized to the corresponding lactone (compound no. 20) in a 78% conversion yield with little enantioselectivity ($E = 15$).

**Biotransformation products of bicyclic ketones.** Table 5 summarizes the Baeyer-Villiger oxidations of bicyclic ketones mediated by *E. coli* BL21(DE3)(pCD201) whole cells. A racemic trans-1-decalone (compound no. 21) containing 10% cis-1-decalone was tested in the whole-cell Baeyer-Villiger oxidation. We found that both enantiomers of trans-1-decalone were good substrates, whereas both cis-1-decalone enantiomers were not. The conversion yield at 20 h reached 80%. This reaction gave two regioisomers of lactone product in a 3.6:1 ratio. The major one (compound no. 22a) gave 33% ee, whereas the minor one

| Substrates | Conversion yield<sup>a</sup> at 20 hrs | Stereoselectivities<sup>b</sup> | Products<sup>c</sup> Isomers content (conv. yield) |
|------------|---------------------------------------|-------------------------------|-----------------------------------------------|
| 21 1-Decalone | 80.0% trans | Two isomers 1 : 7.6 (99%ee) (33%ee) | 22a 78% 22b (80%) |
| 23 cis/trans-2-Decalone | 23.1% cis/trans | Three isomers 1 : 2.1 : 1 (at 20 hrs) | 24a cis- only 70% 24b cis/trans (30.7% at 28 hrs) |
| 25 β-Tetralone | 58.9% | Two isomers 1 : 2.3 | 26a 70% 26b 30% (61.0% at 26 hrs) |
| 27 2-Methyl-1-indone | 28.1% | $E \approx 1$ | 28 (28.7% at 44 hrs) |

<sup>a</sup> Conversion yield was based on the EtOAc-extracted sample determined by normal-phase or chiral-phase GC analysis and referred to an internal standard.

<sup>b</sup> Stereoselectivities refer to regioisomeric lactone ratio, and the given enantiomeric excess values are based on determinations of β-Dex 225 chiral-phase column GC analysis.

<sup>c</sup> Product isomer contents were based on both normal-phase GC-MS and chiral-phase GC analyses.
(compound no. 22b) gave 98% ee based on chiral-GC results. However, the conversion yield did not improve after prolonged incubation.

With 2-decalone (compound no. 23), which was a mixture of 20% of cis-2-decalone and 80% of trans-2-decalone, three regioisomer lactone products were obtained in a total conversion of 23% at 20 h. The cis isomer was completely consumed, and the two enantiomers were oxidized to two alternate lactone regioisomers. Such behavior is similar to the monocyclic ketone system described earlier. In this case, the cis isomer is a better substrate than the trans isomer. β-Tetralone (compound no. 25) and 2-methyl-1-indone (compound no. 27) were also shown to be good substrates for CPDMO, although the stereoselectivity was not high.

**DISCUSSION**

This study describes the cloning, purification, and characterization of CPDMO, a new type I BVMO that contains FAD as a cofactor and uses NADPH as an electron donor (26). CPDMO is a valuable addition to a short but growing list of cloned BVMOs as regio- and/or enantioselective biocatalysts (for a review, see reference 26; for recent reports, see references 5, 6, 16, 17, 25, and 64).

The sequence of CPDMO is characterized by an N-terminal extension of 66 amino acids compared to that of the prototypical CHMO of *Acinetobacter sp.* strain NCIMB 9871 (22, 29). HAPMO (640 amino acids), an aromatic monooxygenase of *P. fluorescens* ACB, is by far the largest BVMO, with an N-terminal extension of about 135 residues (25). This N-terminal portion was deemed important for the structural integrity of the dimeric protein, since deletion of the first 115 amino acids rendered the protein inactive and insoluble (25). In the case of CPDMO, truncation of the 55 N-terminal amino acids in pCpdB-delN resulted in the expression of a soluble but almost inactive protein. Presumably, this resulted in an apoprotein which had lost its ability to bind FAD efficiently (demonstrated by an increased 280/440 nm ratio). Nonetheless, this N-terminal extension in CPDMO, as well as in HAPMO, is expected to constitute an additional domain besides the two-domain architecture, consisting of a FAD-binding and an NADPH-binding domain, found in the three-dimensional structure of a PAMO that originated from the genome of a thermophilic actinomycete, *Thermobifida fusca* (37). It will be interesting to find the possible contribution of the newly identified cluster of charged amino acids to the function of CPDMO or in a related protein.

A second sequence characteristic of CPDMO and its few related proteins is the presence of a 9- or 10-amino-acid insertion between the BVMO fingerprint and the second Rossmann fold (Fig. 2B). According to the three-dimensional structure of PAMO, the fingerprint amino acids constitute a linker region that connects the FAD-binding domain and the NADPH domain (37), and they are not part of the active site. We reckoned that this region may specify some substrate determinants towards the large cyclic ketones. Unfortunately, deletion of seven amino acids in pCpdB-del7 rendered the protein insoluble and inactive. However, a single amino acid substitution in the G242A mutant produced a variant that retained 70% of the wild-type enzyme activity. In the absence of structural information regarding a BVMO that is complexed with its substrate or cofactor (37), we are not able to make any definite statement about the G242A variant at this time.

With reference to the PAMO structure, seven of the eight residues involved in FAD binding are conserved in CPDMO. These amino acids are W112, Y117, D123, Y129, R387, F439, and M500; the corresponding residues in the PAMO sequence are W55, Y60, D66, Y72, R337, F389, and M446, respectively (37). The exception is A442 of PAMO which appears to be replaced by T494 in CPDMO in the present sequence alignment. CPDMO R387 is the equivalent of R337 of PAMO, which is part of the active site and strictly conserved among BVMOs (37). Also, extrapolating from the studies on HAPMO (27) and PAMO, the R280 and K386 of CPDMO are predicted to represent NADPH specificity determinants. In the PAMO structure these residues are R217 and K336 (37).

The most interesting mutant derived from this study is S261A, which led to a significantly higher affinity of the variant towards NADPH, with a *K*ₐ of 8 μM compared to the 24 μM value of the native CPDMO. The former value fits well with the few determined NADPH *K*ₐ values of CPMO, CHMO, and HAPMO that are <3 μM, 7 to 10 μM, and 64 μM, respectively (25, 53, 61). It appeared that in the native CPDMO sequence, a serine substitution at position 261 that is a deviation from the consensus dinucleotide binding motif (GXXGXXG or A) in the second Rossmann fold motif led to a decreased affinity towards the cofactor. In addition, the S261A mutant led to an increased stability by almost doubling its half-life (90 days versus the 50 days of the wild-type enzyme).

CPDMO activity was initially assayed for its conversion of cyclododecanone to lauryl lactone using the conventional NADPH oxidation. As a proof of concept, we have used the state-of-the-art ReactIR 4000 technology that employs mid-infrared spectroscopy and a diamond probe to monitor the progress of this BVMO reaction as a function of time. Previously, Dadd et al. (11) had used a silicon probe of ReactIR to follow the enzyme kinetics involved in nitrile biocatalysis using whole cells of *Rhodococcus rhodochrous* LL100-21. This technique provided excellent quantitative and qualitative real-time data of nitrile biocatalytic reactions that are largely water soluble. As shown in Fig. 5, conversion of the ketone group of cyclododecanone to a lactone by the purified CPDMO is readily monitored at the appropriate wavelength as a function of time. In a separate study, we have followed the bioconversion of cyclododecanone to lauryl lactone in a two-phase bio-reactor and whole-cell system for the preparation of the water-insoluble lactone (J. Yang, M.-J. Lorraine, D. Rho, and P. C. K. Lau, unpublished data). Lauryl lactone is a compound that is not readily available by chemical production (59).

CPDMO is also capable of producing oxacyclohexadecane-2-one, a musk compound responsible for the pleasant odor of angelica root oil used as a fixative in perfumery (trade name Exaltolide [the Merck Index]). However, we are particularly interested in knowing whether CPDMO is capable of producing chiral products, as has been shown for several BVMOs that demonstrated good enantio- or regioselectivity (41, 56, 58). To prove the concept, CPDMO was used to produce a 15-hexadecanolate that represents a rare example of macrolactones known in the literature. (R)-15-hexadecanolate is one of the three active components of a stink bug *Piezodorus hybneri* pheromone that elicits aggregation behavior in males. This
stink bug commonly infests leguminous crops, such as soybeans and kidney beans (36). The two stereoisomers of hexadecanolides are otherwise synthesized by a solvent-intensive chemical route involving at least six steps using the Yamaguchi or Mitsunobu macroalcoholization reaction of a chiral material, (R)-15-hydroxyhexadecanoic acid (33). As it turned out, although a one-step biotransformation is possible, CPDMO was able to make only the nonactive S-stereoisomer, besides giving low enantioselectivity (59% ee; E = 5.8).

Nonetheless, CPDMO displays a wide spectrum of Baeyer-Villiger oxidations of cyclic ketones that includes methyl-substituted short-chain cyclic compounds (C5 and C6) and bicyclic ketones, such as decaalone and β-tetralone. These are poor substrates of the well-characterized Acinetobacter CHMO and CPMO from Comamonas sp. strain NCIMB 9872 (56, 66). CPDMO is particularly useful for the synthesis of both enantiomers of the 7-methyl-2-oxepanone lactone (compound no. 4). CPDMO was able to synthesize 7-methyl-2-oxepanone from 2-methylcyclohexanone (compound no. 3) with an impressive E value of >200 and almost complete (S)-selectivity (99% ee). However, such a good enantioselective result was previously found to be achieved by a recombinant CDMO derived from Rhodococcus SC1 but not seven other BVMOs (34). The enantiomer of 7-methyl-2-oxepanone (compound no. 4) is a desirable chiral building block and substitute for chiral ω-2-hydroxyheptanoic acid that was recently used for the synthesis of a daunome, a phenome that has an antiaging effect in worms (24). However, ω-2-hydroxyheptanoic acid moiety was synthesized from a four-step chemical transformation that is quite expensive and dangerous, requiring regiospecific Grignard-type alkylation of (R)-(+)-propylene oxide with 5-bromo-1-pentene and tetrahydrofuran solvent and later oxidation of the double bond to acid (24). Alternatively, racemic 7-methyl-2-oxepanone (compound no. 4) can be prepared by reaction of the 2-methylcyclohexane (compound no. 3) with m-chloroperbenzoic acid in dichloromethane (39).

In summary, the discovery and purification of CPDMO pave the way for its possible structural determination, knowledge of which will provide great value in further biotechnology development or application, besides serving as a guide for possible targeted changes in the primary structure of CPDMO to increase its biocatalytic potential. Substrate profiling of CPDMO constitutes a quintessential first step towards the possibility of a desirable bioproduction of commercially useful compounds. Recently, Hilker et al. provided the first example of a Baeyer-Villiger oxidation that operated at near-kilogram scale of a (±)-bicyclo[3.2.0]hept-2-en-6-one substrate for the production of optically pure lactones (21).

Finally, this study reiterates the need to isolate and characterize new microorganisms in the environment, not only because we want to enrich the little that we know about microbial diversity or community structure, but also because new microorganisms may have interesting new metabolic or biocatalytic properties (13, 43, 50). No strain, no gain.

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