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Microbial hydroxylation of (*Z*)-2-benzylidene-1-azabicyclo[2.2.2]octan-3-one

John W. Wong * and Michael P. Burns

Bioprocess R&D, Pfizer Central Research, Groton, Connecticut 06340, USA

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Abstract

Microbial hydroxylation of (*Z*)-2-benzylidene-1-azabicyclo[2.2.2]octan-3-one **1** by various species of fungi and actinomycetes occurred regio- and stereoselectively at the 5 position of the quinuclidinone moiety. Most of the organisms produced α-(5*S*)-(*Z*)-2-benzylidene-5-hydroxy-1-azabicyclo[2.2.2]octan-3-one **2** as the major product with enantiomeric excesses ranging from 30% to 84%. The (5β)-hydroxy epimer **3** and the (5α,7α)-dihydroxy derivative **4** were also produced by whole cell biotransformations of **1**.

1. Introduction

The addition of oxygen to organic molecules using biological systems is widely recognized as an important tool for the organic chemist.[1] Microbial hydroxylation is an established technique for the syntheses of steroids[2] and alkaloids,[3] and has been used to prepare derivatives of many complex natural products including ascomycin,[4] artemether,[5] paclitaxel,[6] and quinidine.[7] The utility of microbial hydroxylation is due to the ability of microorganisms to introduce hydroxyl groups at non-activated carbon centers regio- and stereoselectively, a reaction that is difficult to perform by standard chemical methods. However, predicting the reaction site and stereoselectivity of microbial hydroxylations is a major unresolved problem that continues to attract research interest.[8,9] Several groups have studied this problem with various microbial systems and substrates, and active site models have been proposed for *Beauveria bassiana*,[10–12] *Calonectria decora*,[13] and *Absidia blakesleeana*.[14] In these studies, monocyclic and polycyclic substrates are frequently selected as targets for microbial hydroxylation. Microbial hydroxylation may be a useful technique for the selective modification of these compounds since their preparation by standard chemical methods is not straightforward, and the resulting products are often useful intermediates to pharmaceutical compounds or natural products. Two groups, for example, have

* Corresponding author. E-mail: john_w_wong@groton.pfizer.com

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reported on the selective microbial hydroxylation of *N*-substituted 7-azabicyclo[2.2.1]heptanes for the synthesis of epibatidine.[15,16] Microbial hydroxylation has also been used to prepare *N*-substituted 2-azabicyclo[2.2.1]heptan-3-one derivatives for the synthesis of carbocyclic nucleosides.[17,18] Bioconversions of tricyclic compounds have been investigated as a potential method for the syntheses of hydroxylated derivatives, and several reports have been published on the selective microbial hydroxylation of adamantane derivatives.[12,19,20] The hydroxylation of quinidine to (3*S*)-hydroxyquinidine is another example of a tricyclic system that has been selectively modified by whole microbial cells.[7,21,22] This example suggested to us the possibility that optically active quinuclidine derivatives could be prepared by stereoselective microbial hydroxylation. In particular, we were interested in the desymmetrization of a prochiral 1-azabicyclo[2.2.2]octane system by microbial hydroxylation, and selected **1**[23] as the substrate for our studies because it is prochiral, has a chromaphore enabling UV detection, and is readily extracted into organic solvents.

2. Results and discussion

2.1. Microbial hydroxylation of *1*

A screen was conducted to identify microorganisms for the hydroxylation of **1**. Microorganisms were selected based primarily on literature reports, and included species of *Absidia*, *Aspergillus*, *Cunninghamella*, *Penicillium*, and *Streptomyces*. The incubation of **1** with various microbial species resulted in the formation of two mono-oxygenated derivatives, **2** and **3**, that were identified as α- and β-isomers of (*Z*)-2-benzylidene-5-hydroxy-1-azabicyclo[2.2.2]octan-3-one, respectively, by ^{1}H and ^{13}C NMR analyses. The structure and absolute configuration of **2** were confirmed by single-crystal X-ray analysis of the (*S*)-(+)-1-(1- naphthyl)ethylcarbamate derivative **5**. A di-oxygenated derivative, made only by *C. echinulata* ATCC 9244, was identified as (α,α)-(*Z*)-2-benzylidene-5,7-dihydroxy-1-azabicyclo[2.2.2]octan-3-one **4**. Product yields and enantiomeric excesses for selected hits are shown in Table 1. All of the organisms favored the production of **2**, except for *S. albulus* ATCC 12757, which favored the production of **3**.

Table 1
Results of bioconversions of **1** using several microorganisms

Microorganism	Time (days)	% Yield[a]				**2**	
		1	**2**	**3**	**4**	Abs. Con.	e.e. (%)
Cunninghamella echinulata ATCC 8688a	1	41.6	13	0	0	(5*R*)	26
Cunninghamella echinulata ATCC 9244	1	4.7	33.3	1.1	29.8	(5*S*)	30
Mucor plumbeus ATCC 4740	4	5.5	9.5	0	0	(5*S*)	35
Streptomyces griseus ATCC 13273	4	39.9	17.1	1.2	0	(5*S*)	55
Streptomyces punipalus ATCC 3529	4	19.9	31.7	7.1	0	(5*S*)	66
Streptomyces coelicolor ATCC 10147	4	35.8	19.7	2.5	0	(5*S*)	72
Streptomyces albulus ATCC 12757	4	3.8	15	22.9	0	(5*S*)	84
Streptomyces griseus ATCC 12648	1	24.5	33.5	4.7	0	(5*S*)	84
Streptomyces griseus ATCC 22345	1	22.4	31.6	2.1	0	(5*S*)	76
Streptomyces rutgersensis ATCC 3350	4	35.7	5.8	0	0	(5*S*)	71
Streptomyces halstedii ATCC 10897	1	39.8	15.7	1.1	0	nd[b]	nd

[a] yield of **1** calculated as percent of total substrate added; yields of **2**, **3**, and **4** calculated as percent of theoretical based on total substrate added; HPLC quantification using external standards method
[b] not determined

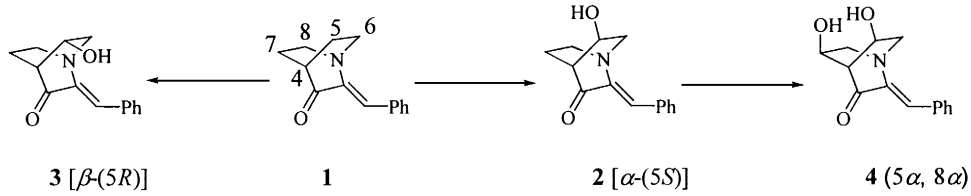
The microbial hydroxylations were stereoselective and favored the formation of α-(5*S*)-**2** (30–84% e.e.) in all organisms except for *C. echinulata* ATCC 8688a. The ability of microbes to hydroxylate **1** was almost exclusively restricted to species of *Streptomyces* and *Cunninghamella*. *Mucor plumbeus* ATCC 4740, previously reported to hydroxylate quinidine to (3*S*)-hydroxyquinidine,[22] was the only organism not belonging to one of these genera to hydroxylate **1**. *Beauveria bassiana* ATCC 7159, frequently reported for its ability to hydroxylate a wide variety of organic substrates including azabicycloalkanes,[8,12,14–17] was included in the screen, but failed to yield any products. This failure may be explained by an enzyme active site model that was initially proposed by Fonken et al.,[10] and later modified by others. This model suggested that an electron rich center in the substrate initially binds to the enzyme's active site followed by hydroxylation at a carbon atom located a specific distance away. In a report based on a study of *N*-phenylcarbamate derivatives, Pietz et al.[12] proposed that *B. bassiana* could only hydroxylate substrates that had suitable hydrogen atoms at least 5.0 Å away from the electron rich center. In **1**, the 5α and 5β hydrogen atoms are located only 4.4 and 3.4 Å, respectively, from the carbonyl oxygen, thus making this compound unsuitable for microbial hydroxylation by *B. bassiana*, according to the refined model proposed by Pietz et al. While this model appears to account for the inability of *B. bassiana* to hydroxylate **1**, predicting the selectivity of this organism may not be straightforward, as a recent study by Holland[8] suggests the presence of up to four distinct hydroxylase activities.

Flask and fermentor scale bioconversions were carried out to provide material for structure elucidation. Compounds **2** and **3** were produced in fermentor cultures of *Streptomyces albulus* ATCC 12757. Compound **2** was also isolated from fermentor cultures of *Streptomyces griseus* ATCC 13273 and used for the preparation of **5**. Bioconversion of **1** to **4** was carried out in flask cultures containing washed cells of *C. echinulata* ATCC 9244 resuspended in phosphate buffer. Compounds **2** and **3** were also produced in flask culture bioconversions with *Streptomyces punipalus* ATCC 3529. However, attempted recovery of these compounds from the *S. punipalus* bioconversion resulted in isolation of 1-phenyl-6,7-dihydro-5*H*-indolizin-8-one **6**. Compound **6** was identified by ^{1}H and ^{13}C NMR analyses, and was likely to have been produced from the oxygenated bioconversion products as a result of exposure to high pH during extraction. Compound **6** was not detected in any of the other bioconversion product mixtures isolated by solvent extractions at neutral or slightly acidic pH.

2.2. Identification of bioconversion products **2**, **3**, and **4**

Compounds **2** and **3** were isolated in 17.6% and 6.8% yields, respectively, from fermentor cultures of *Streptomyces albulus* ATCC 12757. Mass spectral analyses by APCI^{+} mass spectrometry revealed MH^{+} ions at 230 for **2** and **3**, indicating that these were oxygenated derivatives. Compounds **2** and **3** were further characterized as the α- and β-5-hydroxy derivatives of **1** (Fig. 1), respectively, by NMR analyses. Carbon and proton NMR signal assignments were supported by data from ^{1}H COSY, HMQC, and ^{13}C DEPT experiments. The ^{13}C DEPT spectra of **2** and **3** displayed distinct signals for three methylene carbons and two aliphatic methine carbons in the upfield region, indicating that hydroxylation had occurred at one of the methylene groups on **1**. Chemical shifts for the new methine carbon signals were observed at δ 66.70 ppm and δ 70.95 ppm for **2** and **3**, respectively, consistent with values expected for oxymethine carbon signals. The positions of hydroxylation were indicated by evidence obtained through ^{1}H COSY experiments. In the ^{1}H COSY spectrum for **2**, a strong correlation between the oxymethine proton signal at δ 4.31 ppm and the bridgehead proton signal at δ 2.81 ppm indicated that hydroxylation had occurred at position 5. Similarly, the ^{1}H COSY spectrum for **3** revealed a strong correlation between the signal for the oxymethine proton at δ 4.38 ppm and the bridgehead proton signal at δ 2.78 ppm. Thus the NMR data indicated that **2** and **3** were epimeric 5-hydroxy derivatives of **1**. In Fig. 1, the absolute

configuration shown for **2** is that of the (+)-isomer produced in greatest abundance by all except one of the microbes used in our screen. The absolute configuration shown for **3** is that of the (−)-isomer produced in greatest abundance in a fermentor scale bioconversion by *Streptomyces albulus* ATCC 12757.



3 [*β*-(5*R*)] **1** **2** [*α*-(5*S*)] **4** (5*α*, 8*α*)

Fig. 1. Microbial hydroxylation products of **1**

The relative configurations of the hydroxyl group in **2** and **3** were assigned by comparative analyses of carbon and proton chemical shifts for the C7 methylene groups. Chemical shifts for the C7 methylene carbons in **2** and **3** were shifted 8.40 ppm and 3.13 ppm upfield, respectively, relative to the resonance of the C7 carbon in **1**. Based on these chemical shift differences, **2** and **3** were assigned α- and β-configurations, respectively. This method was also used by Carroll et al.[7] to assign the absolute configuration of 3-hydroxyquinidine, produced in a biotransformation of quinidine with *Streptomyces griseus* ATCC 13273.

Further confirmation for the relative configurations of **2** and **3** was obtained by comparison of the chemical shifts for the methylene protons gamma to C5. Due to the proximity of the hydroxyl group, the C7 proton of **2**, *syn* to the hydroxyl group, should experience greater deshielding compared to the analogous proton in **3**. Chemical shift differences of 0.49 ppm and −0.24 ppm for the C7 protons of **2**, and shift differences of 0.04 ppm and −0.10 ppm for the C7 protons of **3**, support our relative configuration assignments based on ^{13}C NMR data. Thus proton and carbon NMR data supported our assignments of α-5-hydroxy and β-5-hydroxy configurations for **2** and **3**, respectively.

The results of our screen (Table 1) showed that microbial hydroxylation of **1** favored the production of **2** in 10 of 11 organisms, and was the only bioconversion product detected for *C. echinulata* ATCC 8688a, *M. plumbeus* ATCC 4740, and *S. rutgersensis* ATCC 3350. To determine whether these microbial hydroxylations were stereoselective, we characterized the enantiomeric compositions of the microbial products by chiral HPLC. Chiral HPLC on a Chiracel® OD column was chosen to determine e.e. since this method resolved the enantiomers of **2** and **3** simultaneously, without purification or derivatization. Fig. 2 shows a chromatogram from a Chiracel® OD separation of the product from the bioconversion of **1** by *Streptomyces punipalus* ATCC 3529. Samples of the (+)- and (−)-enantiomers of **2** and **3** were separately collected by HPLC on a Chiracel® OD column, and their structures confirmed by ^{1}H and ^{13}C NMR spectroscopy and optical rotation measurements. Chiral HPLC analyses were performed on the microbial screen products and enantiomeric excesses for **2** are shown in Table 1. The results showed that microbial hydroxylation of **1** was stereoselective for the earlier eluting (+)-enantiomer in all cases except for one, with enantiomeric excesses ranging from 30% to 84%. Due to the low yields of **3** produced by the microorganisms in our screen, enantiomeric excesses were only determined for *S. albulus* ATCC 12757. In the test tube scale bioconversion performed for the screen, *S. albulus* ATCC 12757 produced the earlier eluting (+)-enantiomer in 54% e.e. Interestingly, this organism produced the (−)-enantiomer in 46% e.e. during the fermentor scale bioconversion.

The absolute configuration of the (+)-enantiomer of **2** was determined as (5*S*) by single-crystal X-ray analysis on the carbamate derivative **5**. Compound **5** was prepared by the reaction of (*S*)-(+)-1-(1-naphthyl)ethylisocyanate with **2** isolated from a bioconversion with *Streptomyces griseus* ATCC 13273. Suitable crystals were obtained by slow evaporation from ethyl acetate. Fig. 3 shows an ORTEP drawing

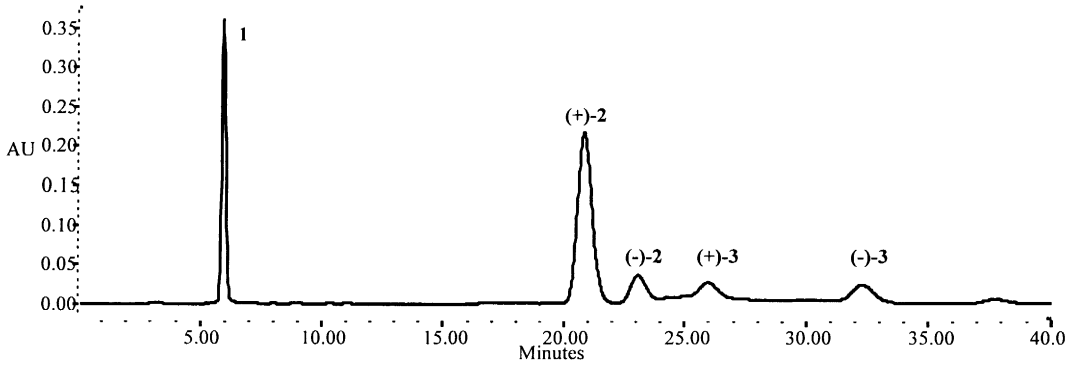


Fig. 2. Chromatogram of Chiracel® OD separation of product from bioconversion of **1** by *Streptomyces punipalus* ATCC 3529. The sample was taken 4 days after substrate addition

of the X-ray crystal structure of **5**. The absolute configurations of (+)-**3** and (−)-**3** were determined as (5*S*) and (5*R*), respectively, by ^{1}H NMR analyses of the Mosher esters prepared with (*R*)-(−)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride.

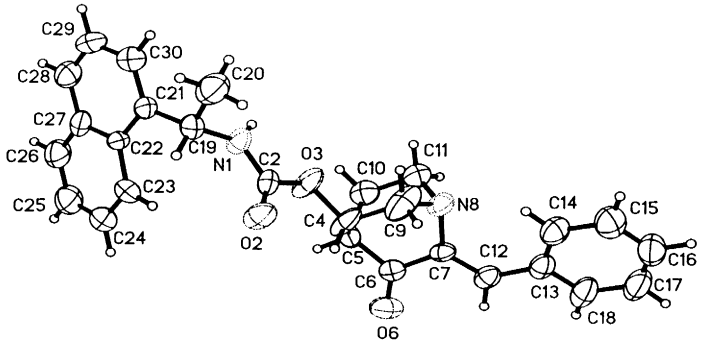


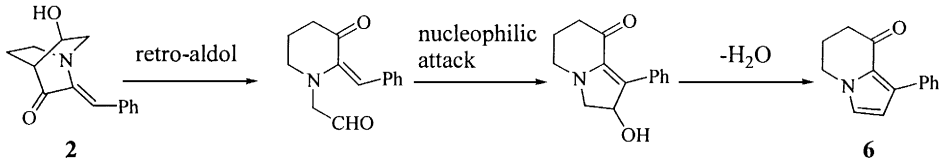
Fig. 3. ORTEP drawing of carbamate derivative **5**

While our microbial screen revealed the production of **2** and **3** in a number of *Cunninghamella* and *Streptomyces* spp., compound **4** was observed as the product of only one bioconversion. *Cunninghamella echinulata* ATCC 9244 produced compounds **2**, **3**, and **4** in a ratio of 30:1:27 after incubation with substrate for 1 day. The structure of compound **4** was proposed as the (5α,7α)-dihydroxy derivative of **1** by mass and NMR spectroscopic analyses. Mass spectroscopic analysis of **4** revealed an MH^{+} ion at 246, indicative of the addition of two oxygens. Since **2** was also a major product of the bioconversion, we suspected that **4** was formed by the oxygenation of **2**. Evidence for a symmetrically substituted diol structure was observed in the ^{13}C DEPT spectrum for **4**. The upfield region of this spectrum contained just two signals for methine carbons (δ 54.59 and δ 67.58 ppm) and one signal for a methylene carbon (δ 58.81 ppm), compared to the spectra for **2** and **3** which contained five carbon resonances in the same region. This simplification in the upfield region of the ^{13}C spectrum for **4** could be explained by a symmetrical dihydroxylation of the quinuclidine nucleus. Evidence for (5α,7α) configurations of the hydroxyl groups was obtained in the close agreement between the chemical shifts of the oxymethine carbons in **2** and **4**. The downfield region of the ^{13}C spectrum of **4** was very similar to the analogous regions in the spectra of compounds **1**, **2**, and **3**, indicating that no changes had occurred in the aromatic and vinylic portions of the structure. Further support for the diol structure was obtained from the ^{1}H NMR spectrum of **4**. Symmetrical substitution of the quinuclidine nucleus was also indicated by the proton NMR spectrum that revealed only four signals for the quinuclidine protons. The proton spectra for **2** and

3, on the other hand, showed discrete signals for all eight protons directly attached to the quinuclidine nucleus. The proposed diol structure was directly supported by ^{1}H COSY correlations between the two-proton signal at δ 4.34 ppm, assigned to oxymethine protons at positions 5 and 7, and the signals for the bridgehead methine proton and the four methylene protons.

*2.3. Identification of 1-phenyl-6,7-dihydro-5*H*-indolizin-8-one **6***

Incubation of **1** in flask cultures of *S. punipalus* resulted in the formation of **2** and **3**. However, after extraction of crude product from the fermentation broth, we observed the presence of a novel compound **6** that was not observed during the bioconversion. Also, the yields of **2** and **3** in the crude product mixture were significantly decreased from the levels that were estimated prior to extraction. These observations suggested that **6** was formed from one or both monooxygenated bioconversion products, probably due to exposure to pH 10 during extraction. A sample of **6** was isolated by HPLC and its structure proposed as the indolizin derivative shown in Scheme 1, on the basis of mass spectral and NMR analyses. Mass spectroscopic analysis of **6** revealed an MH^+ ion at 212, indicating that **6** was formed from **2** or **3** with the loss of water. Loss of the hydroxyl group was confirmed by the absence of an oxymethine signal in the ^{1}H NMR spectrum. Furthermore, the ^{1}H NMR spectrum also lacked a signal for a bridgehead methine proton, indicating that the quinuclidinone ring system was no longer intact. Major structural features supported by ^{1}H COSY data included the presence of three contiguous methylene groups and two vinyl protons on adjacent carbons. The indolizin structure proposed for **6** is consistent with the mass and NMR spectral data, and was probably formed beginning with a base catalyzed retro-aldol opening of the hydroxyl-substituted bridge of the quinuclidine system to give an aldehyde. Formation of the five-membered ring could result from an intramolecular nucleophilic attack on the aldehyde moiety followed by elimination of water, as proposed in Scheme 1.



2 **6**

Scheme 1.

3. Conclusion

Our results have shown that (*Z*)-2-benzylidene-1-azabicyclo[2.2.2]octan-3-one **1** was desymmetrized by microbial hydroxylation with species of *Streptomyces*, *Cunninghamella*, and one species of *Mucor*. Hydroxylation occurred regioselectively at the 5 position, and was stereoselective for the α-(5*S*)-isomer in all but one of the organisms for which stereoselectivity was determined. Some of the organisms gave moderately good yields of **2** considering the bioconversions were unoptimized. Only one organism, *Cunninghamella echinulata* ATCC 9244, produced the dihydroxylated derivative **4**. Although our microbial screen included organisms from several genera, it is interesting to note that hydroxylation was generally restricted to species of *Streptomyces*. The inability of *Beauveria bassiana* ATCC 7159 to hydroxylate **1** appears to support the active site model[12] that proposes a minimum of 5 Å for the distance between the electron rich atom that binds to the enzyme active site and the hydrogen atom to be replaced by oxygen.

4. Experimental

4.1. General

Melting points were determined by differential scanning calorimetry on a Perkin–Elmer DSC-7 and reported as Tmax for **2**, **4**, and **5**. Thermogravimetric analysis on a Perkin–Elmer TGA-7 was used to determine the melting point for **3**. ^{1}H NMR spectra were obtained in $CDCl_3$ at 400 MHz on a Varian Unity Plus spectrometer with solvent as the internal standard. ^{13}C NMR spectra were recorded in $CDCl_3$ at 100 MHz. Chemical shift values are reported in parts per million (ppm), and coupling constants given in hertz. Abbreviations used for NMR signals are as follows: s, singlet; d, doublet; t, triplet; and m, multiplet. High performance liquid chromatography (HPLC) was performed on a Waters system consisting of a 600 controller, 996 photodiode array detector and 717 autosampler. Reverse phase analyses were performed on a 3.9×150 mm Symmetry® C18 (Waters) column by gradient elution with mixtures of 20 mM NaH_2PO_4 and acetonitrile (ACN). Detection was carried out at 299 nm. Optical purities of bioconversion products were determined by HPLC on a 4.6 mm id×250 mm Chiracel® OD column (Chiral Technologies) eluted isocratically with a mixture of hexane:ethanol, 98:2, containing 0.025% TFA. Semi-preparative separations were performed on the Waters HPLC system described above, equipped with a 19 mm id×300 mm Symmetry® C18 column (Waters), and eluted isocratically with a mixture of 0.02% TFA in 10 mM ammonium acetate:ACN, 58:42. Semi-preparative HPLC separations were also performed on a Waters PrepLC 4000 system consisting of a Waters 4000 system controller, a Waters 486 tunable absorbance detector, and equipped with a 25 mm id×210 mm NovaPak C18 radial compression cartridge (Waters). This system was eluted with a mixture of 20 mM NaH_2PO_4:ACN, 70:30. Optical rotations were determined on ethanol solutions in a 1 dm cell on a Perkin–Elmer 241 spectropolarimeter. Routine mass spectral analyses were performed on a Micromass platform II spectrometer with an atmospheric pressure chemical ionization ($APCI^+$) source. High resolution mass spectral analyses (HRMS) were obtained by M-Scan, Inc., West Chester, PA, on a VG Analytical ZAB 2SE high field mass spectrometer. Elemental analyses were performed by Schwarzkopf Microanalytical Laboratory, Woodside, NY. Yields of products are given as a percentage of the theoretical value.

4.2. Starting material

(*Z*)-2-Benzylidene-1-azabicyclo[2.2.2]octan-3-one **1** was prepared as previously described.[23] ^{1}H NMR: δ 2.00 (dt, 7.8, 2.9 Hz, four methylene protons on C5 and C7), 2.61 (m, H4), 2.97 (dt, 13.9, 7.8 Hz, H6a, H8a), 3.14 (dt, 13.9, 7.8 Hz, H6b, H8b), 7.00 (s, H1′), 7.32–7.37 (m, H4′, H5′, H6′), 8.00 (d, 6.8 Hz, H3′, H7′); ^{13}C NMR: δ 25.88 (C5, C7), 40.28 (C4), 47.48 (C6, C8), 125.13 (C1′), 128.40 (C4′, C6′), 129.55 (C5′), 132.15 (C3′, C7′), 144.71 (C2), 206.44 (C3).

4.3. Biotransformation conditions

Microorganisms were obtained from commercial collections and maintained as glycerol suspensions of spores or vegetative cells at −70°C, or on slant cultures at 4°C. Microbes were cultured in a medium comprised of dextrose (20 g/L), soyflour (5 g/L), yeast extract (5 g/L), NaCl (5 g/L), and K_2HPO_4 (5 g/L) in deionized water, and adjusted to pH 7.0 before autoclaving. The microbial screen was conducted in glass tubes (16 mm×125 mm) containing 2.5 mL of medium inoculated with glycerol suspensions of spores or vegetative cells from various microorganisms and agitated on a rotatory shaker at 210 rpm

and 29°C. After 48 h, a solution of **1** in dimethylsulfoxide (DMSO) was added to cultures to give a concentration of 0.2 g/L, and incubation continued for a period of 1 to 4 days.

Preparative biotransformations were carried out with *Streptomyces albulus* ATCC 12757 and *Streptomyces griseus* ATCC 13273 cultures in two stage protocols. In the first stage *S. albulus* cultures were grown in ten 300 mL flasks each containing 30 mL of medium. These flasks were inoculated with a glycerol suspension of spores or vegetative cells and incubated on a rotatory shaker at 210 rpm and 29°C for 48 h. The contents of these flasks were combined and used to inoculate two fermentor cultures, each containing 8 L of medium. Fermentor cultures were incubated at 29°C, 400 rpm agitation, and 4 L/min aeration for 48 h, after which bioconversion was initiated by addition of a DMSO solution of **1**. The *S. albulus* cultures were incubated for 66 h after substrate addition. The first stage of the *S. griseus* cultures were grown in Fernbach flasks containing 800 mL of medium. Three flasks were inoculated from slant cultures and incubated at 29°C for 24 h. Three fermentors containing 8 L of medium were each inoculated with a single flask culture, and operated at 29°C, 600 rpm agitation, and 6 L/min aeration. After 24 h, bioconversion was started by addition of a DMSO solution of **1**, and incubation continued for 30 h. Preparative biotransformations were also performed in flask cultures with washed cells of *Cunninghamella echinulata* ATCC 9244 resuspended in phosphate buffer. Three 1 L flasks containing 250 mL of medium were inoculated with a glycerol suspension of *C. echinulata* ATCC 9244 spores and incubated on a rotatory shaker (210 rpm) at 27°C for 72 h. Cells were collected from each flask by filtration, washed with an aqueous solution of NaCl (5 g/L) and K_2HPO_4 (5 g/L), and resuspended in 250 mL of phosphate buffer (100 mM, pH 7.0) containing glucose (5 g/L) and TWEEN 80 (5 g/L). Biotransformation was initiated by addition of a DMSO solution of **1** to each flask, and incubation continued for 24 h.

4.4. Extraction and analyses of microbial screen samples

The whole broths from the microbial screen fermentations were extracted with ethyl acetate, dried under nitrogen, and reconstituted in a mixture of ACN:H_2O, 4:1, for reverse phase HPLC analyses on a Symmetry® C18 4.6 mm id×150 mm column. Selected samples were concentrated to dryness under a stream of nitrogen and reconstituted in ethanol for HPLC analyses on a Chiracel® OD column.

4.5. Isolation and purification of biotransformation products

Biotransformation products **2** and **3** were isolated from *Streptomyces albulus* fermentation broth with a hydrophobic resin. Cells were removed from whole broth by centrifugation, and the supernate passed over a column of XAD-4 resin (Rohm & Haas). The resin was eluted with a 1:1 mixture of methanol:H_2O followed by ethyl acetate. The ethyl acetate fraction was concentrated under reduced pressure and subjected to semi-preparative HPLC on a 19 mm id×300 mm Symmetry C18 column to give **2** and **3**. These compounds were crystallized from methanol or a mixture of ethyl acetate:hexanes. Further separations of compounds **2** and **3** were carried out by HPLC on a 4.6 mm id×250 mm Chiracel® OD column to yield the individual enantiomers. Biotransformation product **2** was also recovered from *Streptomyces griseus* fermentation broth using XAD-4 resin. Cell-free supernate was passed over an XAD-4 resin column and eluted with a step gradient of methanol in water. Fractions containing the desired product were concentrated to remove methanol and extracted with ethyl acetate to yield the crude product. Crystallization from methanol afforded **2**. Compound **4** was isolated from the *Cunninghamella echinulata* ATCC 9244 washed cell bioconversion by ethyl acetate extraction of the cell-free filtrate. The ethyl acetate extract was concentrated under reduced pressure and partitioned between methanol

and hexanes. The methanol fraction was concentrated under reduced pressure and subjected to semi-preparative HPLC on a 25×210 mm Novapak® C18 radial compression cartridge to afford **4**.

4.6. *Bioconversion of **1** to **2** and **3** by* Streptomyces albulus *ATCC 12757*

Compound **1** (3.2 g) was incubated in two 8 L fermentor cultures of *S. albulus*. After 66 h incubation, biotransformation products were extracted from the culture broth with XAD-4 resin and separated by reverse phase HPLC to afford two products. Compound **2** was isolated in a yield of 17.6% (605 mg); m.p.=191.1°C (crystallized from methanol); HRMS: calcd for $C_{14}H_{15}NO_2+H^+$ 230.1181, found 230.1191. Anal. $C_{14}H_{15}NO_2$ (229.27), calcd %: C, 73.34, H, 6.59, N, 6.11; found%: C, 73.31, H, 6.48, N, 6.16. ^{1}H NMR: δ 1.78 (ddddd, 13.5, 10.7, 5.7, 2.8, 1.4 Hz, H7β), 2.11 (d, 3.4 Hz, OH), 2.50 (dddd, 13.5, 10.5, 5.0, 3.0 Hz, H7α), 2.81 (m, H4), 2.92 (dd, 14.7, 3.4 Hz, H6β), 2.98 (dddd, 13.5, 10.7, 5.0, 2.3 Hz, H8β), 3.27 (ddd, 13.5, 10.5, 5.7 Hz, H8α), 3.33 (ddd, 14.7, 8.3, 2.3 Hz, H6α), 7.01 (s, H1′), 7.33–7.38 (m, H4′, H5′, H6′), 8.00–8.02 (m, H3′, H7′); ^{13}C NMR: δ 17.46 (C7), 47.15 (C8), 49.11 (C4), 57.87 (C6), 66.68 (C5), 125.93 (C1′), 128.51 and 129.92 (C4′, C5′, C6′) 132.27 (C3′, C7′), 133.57 (C2′), 143.46 (C2), 204.33 (C3). Separation of **2** on a Chiracel® OD column yielded the earlier-eluting (+)-**2**, $[\alpha]_D^{23}$=+35.7 (c 0.35, methanol), and (−)-**2**, $[\alpha]_D^{23}$=−37.8 (c 0.12, methanol). Compound **3** was isolated in a yield of 6.8% (234 mg); m.p.=138.4–147.7°C (crystallized from hexane:ethyl acetate); HRMS: calcd for $C_{14}H_{15}NO_2+H^+$ 230.1181, found 230.1167. ^{1}H NMR: δ 1.90 (dddd, 13.7, 10.3, 6.4, 2.6 Hz, H7β), 2.03 (dddd, 13.7, 10.0, 5.2, 3.9 Hz, H7α), 2.62 (d, 2.4 Hz, OH), 2.78 (m, H4), 2.87 (ddd, 14.3, 4.8, 2.0 Hz, H6α), 2.91 (ddd, 10.0, 6.4, 2.0, H8β), 2.99 (ddd, 13.2, 10.3, 5.2 Hz, H8α), 3.46 (dd, 14.3, 7.9 Hz, 6β), 4.38 (m, H5), 7.02 (s, H1′), 7.32–7.38 (m, H4′, H5′, H6′), 7.99–8.03 (m, H3′, H7′); ^{13}C NMR: δ 22.75 (C7), 46.54 (C8), 48.70 (C4), 58.11 (C6), 70.94 (C5), 125.24 (C1′), 128.42 and 129.74 (C4′, C5′, C6′), 132.34 (C3′, C7′), 133.73 (C2′), 143.97 (C2), 203.80 (C3). Separation of **3** on a Chiracel® OD column yielded the earlier-eluting (+)-**3**, $[\alpha]_D^{23}$=+33.9 (c 0.26, methanol), and (−)-**3**, $[\alpha]_D^{23}$=−34.2 (c 0.40, methanol).

4.7. *Bioconversion of **1** to **2** by* Streptomyces griseus *ATCC 13273*

Compound **1** (4.8 g) was incubated in three 8 L fermentor cultures of *S. griseus*. After 24 h, the biotransformation product was recovered from the cell-free fermentation broth with XAD-4 resin. Crystallization from methanol afforded 1.05 g (20.4% yield) of (S)-(+)-**2** (76% e.e.). ^{1}H and ^{13}C NMR were identical to spectra obtained for **2** isolated from the *S. albulus* fermentation.

4.8. *Bioconversion of **1** to **4** by* Cunninghamella echinulata *ATCC 9244*

A total of 150 mg of **1** was incubated in three flasks containing washed cells of *C. echinulata* ATCC 9244. After 23 h, the biotransformation product was recovered by ethyl acetate extraction of the cell-free fermentation broth, and purified by HPLC to afford 31 mg of **4** (17.9% yield); m.p.=175.3°C (crystallized from methanol); HRMS: calcd for $C_{14}H_{15}NO_3+H^+$ 246.1130, found 246.1135. Anal. $C_{14}H_{15}NO_3$ (245.27), calcd %: C, 68.56, H, 6.16, N, 5.71; found %: C, 68.15, H, 6.08, N, 5.69. ^{1}H NMR: δ 3.17 (dd, 14.1, 3.7 Hz, H6β, H8β), 3.23 (t, 3.2 Hz, H4), 3.54 (dd, 14.1, 8.5 Hz, H6α, H8α), 4.07 (br s, 2 OH), 4.34 (m, H5, H7), 7.01 (s, H1′), 8.00–8.02 (m, H3′, H7′), 7.36–7.38 (m, H4′, H5′, H6′); ^{13}C NMR: δ 54.49 (C4), 58.73 (C6, C8), 67.48 (C5, C7), 127.02 (C1′), 128.61 (C4′, C6′), 130.36 (C5′), 132.40 (C3′, C7′), 133.11 (C2′), 142.27 (C2), 200.50 (C3).

4.9. Single-crystal X-ray structure determination of (S)-(+)-1-(1- naphthyl)ethylcarbamate derivative **5**

Compound **2** (50 mg, 0.22 mmol), isolated from the bioconversion of **1** by *S. griseus* ATCC 13273, was combined with (*S*)-(+)-1-(1-naphthyl)ethylisocyanate (47 mg, 0.24 mmol) and dimethylaminopyridine (12 mg, 0.1 mmol) in 5 mL of anhydrous dichloromethane. The reaction was stirred under nitrogen at 22°C for 24 h. Water was added to the reaction mixture and the organic phase separated and dried over anhydrous magnesium sulfate. The solvent was removed under reduced pressure and the residue chromatographed on a 40 mm×200 mm Novapak C18 radial compression cartridge to afford 64 mg of **5** (69% yield); m.p.=175.0°C (crystallized from ethyl acetate:hexane); HRMS: calcd for $C_{27}H_{26}N_2O_3$+H^+ 427.2022, found 427.2012; ^{1}H NMR: δ 1.68 (d, 6.0 Hz, 3H), 1.75 (m, 1H), 2.24 (m, 1H), 2.97 (m, 3H), 3.16 (m, 1H), 3.43 (m, 1H), 5.09 (m NH and 1H), 5.66 (m, 1H), 7.02 (s, 1H), 7.35 (m, 3H), 7.44–7.57 (m, 4H), 7.80 (d, 7.9 Hz, 1H), 7.87 (m, 2H), 8.00 (m, 1H), 8.10 (d, 8.3 Hz, 1H). Crystallographic data for **5**: $C_{27}H_{26}N_2O_3$, *M*=426.52, 0.10×0.26×0.34 mm, *a*=6.112 (3), *b*=10.390 (3), *c*=35.64 (1) Å^3, V=2262.9 (1) Å^3, ρ_{calc}=1.252 g cm^{-3}, μ=6.55 cm^{-1}, *Z*=4, needles, space group $P2_12_12_1$, 1391 independent and 1110 observed reflections (sin θ/λ=0.5 Å^{-1}; $I>3.0\,\sigma$), *R*=0.049. All data sets were collected on a Siemens R3RA/V diffractometer at room temperature. Atomic scattering factors were taken from the *International Tables for X-Ray Crystallography*.[24] All crystallographic calculations were facilitated by the SHELXTL system.[25] A trial structure was obtained by direct methods and refined routinely. Hydrogen positions were calculated wherever possible. The methyl hydrogens were located by difference Fourier techniques. The hydrogen parameters were added to the structure factor calculations but were not refined. The shifts calculated in the final cycle of least squares refinement were all less than 0.1 of their corresponding standard deviations. The refined structure was plotted using the SHELXTL plotting package (Fig. 3).

4.10. Preparation of Mosher esters of **3**

Mosher esters were prepared with compound **3** isolated from the *Streptomyces albulus* bioconversion described in Section 4.6. To a 2 mL reaction vial equipped with a septum and a magnetic stir bar was added **3** (14.9 mg, 0.065 mmol), $CHCl_3$ (1 mL), triethylamine (26 μL, 0.2 mmol), and a crystal of DMAP. (*R*)-(−)-α-Methoxy-α-(trifluoromethyl)phenylacetyl chloride (32.3 mg, 0.128 mmol) was then added as a solution in $CHCl_3$ (0.3 mL) and the reaction mixture was stirred overnight at room temperature. The reaction mixture was then filtered through a 5 g silica Sep-Pak® cartridge (Waters) and eluted with a mixture of ethyl acetate:hexanes (1:3, v/v) to give the Mosher esters of **3**. This material was subjected to ^{1}H NMR in $CDCl_3$ without further purification. The absolute configuration assignment was based on the chemical shift of the 6β protons for the major (*R*,*R*)-diastereomer (δ 3.56) and the (*R*,*S*)-diastereomer (δ 3.63).

4.11. Isolation of 1-phenyl-6,7-dihydro-5H-indolizin-8-one **6**

A total of 200 mg of **1** was added to four flask cultures of *Streptomyces punipalus* ATCC 3529. After 84 h incubation, the fermentation broth was filtered to remove cells, adjusted to a pH of 10 with 4N sodium hydroxide, and extracted with ethyl acetate. The crude product was separated by semi-preparative HPLC on a 7.8 mm id×300 mm Novapak C18 column eluted with a mixture of 20 mM NaH_2PO_4:ACN (65:35) to give 10 mg of compound **6**; APCI$^+$ MS: 212 (MH^+); ^{1}H NMR: δ 2.28 (m, 2H), 2.61 (dd, 7.0, 5.9 Hz, 2H), 4.14 (t, 5.8 Hz, 2H), 6.33 (d, 2.6 Hz, 1H), 6.85 (d, 2.6 Hz, 1H), 7.27 (m, 1H), 7.35 (m, 2H), 7.63 (m, 2H); ^{13}C NMR: δ 23.35, 37.42, 45.97, 111.65, 125.20, 127.14, 127.76, 129.21, 132.74, 135.07, 186.76.

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