Recrystallization from water gave the analytical sample of **6** (mp 258–260 °C) which was dried under vacuum at room temperature: $^1$H NMR ($Me_2SO$-$d_6$–$D_2O$, $Me_4Si$) δ 8.50, 8.42, 8.01, 7.93 (2 H-6′ of pyrimidine and H-2 and H-8 of hypoxanthine), 5.44, 5.04 (2 $CH_2N$), 3.74, 3.72 (2 $NCH_3$), 2.58 (2 $CCH_3$); $^{13}$C NMR ($Me_2SO$-$d_6$, $Me_4Si$) δ 162.2, 162.0, 161.3, 161.0, 156.8, 154.0 (6 s), 147.4, 147.0, 146.3 (3 d), 114.7, 111.4, 111.2 (3 s), 42.7, 41.8 (2 t, $NCH_2$), 41.7, 41.6 (2 q, NMe), 21.4, 21.3 (2 q, CMe); one CH signal in the δ 146–147 region either overlapped another or had insufficient intensity to be detected. Anal. Calcd for $C_{19}H_{24}N_{10}O_9Cl_2 \cdot H_2O$: C, 35.47; H, 4.39; N, 21.77. Found: C, 35.42; H, 4.32; N, 21.97.

**1,7-Bis[(4-amino-1,2-dimethyl-5-pyrimidinio)methyl]guanine Diperchlorate (7).** A mixture of 0.295 g (1.05 mmol) of guanosine, 1.0 g (2.1 mmol) of 1′-methylthiaminium diperchlorate,[8] 0.5 mL of 2,4,6-trimethylpyridine, and 30 mL of methanol was heated at reflux for 16 h. The solvent then was removed on an evaporator, and the residue was washed with absolute ethanol and was recrystallized twice from 0.1 M perchloric acid. The third recrystallization from water gave 0.18 g (0.27 mmol, 26%) of the analytical sample of **7** (mp 246–248 °C dec) which was dried at room temperature under vacuum: $^1$H NMR ($Me_2SO$-$d_6$–$D_2O$, $Me_4Si$) δ 8.35, 8.05, 7.88 (H-8 guanine and 2 H-6′ of pyrimidine), 5.42, 4.98 (2 $NCH_2$), 3.88 (2 $NCH_3$), 2.65 (2 $CCH_3$); UV (pH 6) 219 nm (log ε 4.48), 249 (4.49). Anal. Calcd for $C_{19}H_{25}N_{11}O_9Cl_2 \cdot H_2O$: C, 34.66; H, 4.44; N, 23.40. Found: C, 34.60; H, 4.42; N, 23.39.

**7-[(4-Amino-1,2-dimethyl-5-pyrimidinio)methyl]xanthine Perchlorate (8).** A mixture of 0.394 g (1.39 mmol) of xanthosine, 1.0 g (2.1 mmol) of 1′-methylthiaminium diperchlorate,[8] 0.5 mL of 2,4,6-trimethylpyridine, and 20 mL of methanol was heated at reflux for 2.5 h. The precipitated white solid was filtered and washed with absolute ethanol. Two recrystallizations from boiling 0.1 M perchloric acid (with filtration of undissolved material) gave 0.30 g (0.77 mmol, 55%) of the analytical sample of **8** (mp >300 °C) which was dried at room temperature under vacuum: $^1$H NMR ($Me_2SO$-$d_6$–$D_2O$, $Me_4Si$) δ 8.13 (H-8 and H-6′), 5.35 ($CH_2N$), 3.77 ($NCH_3$), 2.60 ($CCH_3$); $^{13}$C NMR ($Me_2SO$-$d_6$, $Me_4Si$) δ 162.2, 161.1, 155.7, 151.1, 149.5 (5 s), 147.2, 142.7 (2 d), 111.1, 106.3 (2 s), 42.2 (t, $CH_2N$), 41.8 (q, NMe), 21.5 (q, CMe). Anal. Calcd for $C_{12}H_{14}N_7O_6Cl$: C, 37.17; H, 3.64; N, 25.29. Found: C, 37.16; H, 3.62; N, 25.26.

**1,3-Bis[(4-amino-1,2-dimethyl-5-pyrimidinio)methyl]uracil Diperchlorate (9).** A mixture of 0.112 g (1.00 mmol) of uracil, 1.0 g (2.1 mmol) of 1′-methylthiaminium diperchlorate,[8] 0.5 mL of 2,4,6-trimethylpyridine, and 20 mL of methanol was heated at reflux for 16 h. The white solid was collected and washed with absolute ethanol to give 0.20 g (0.34 mmol, 34%) of crude product. Recrystallization from water gave the analytical sample of **9** (mp 258–260 °C) which was dried at room temperature under vacuum: $^1$H NMR ($Me_2SO$-$d_6$) 9.19, 8.40 (4 NH), 8.12 (2 H-6′), 7.87 (d, H-6, $J$ = 8 Hz), 6.01 (d, H-5, $J$ = 8 Hz), 4.78 (2 $CH_2N$), 3.78 (2 $NCH_3$), 2.58 (2 $CCH_3$); $^{13}$C NMR ($Me_2SO$-$d_6$, $Me_4Si$) δ 162.8, 161.8, 161.7, 161.2, 151.4 (5 s), 145.7, 145.5, 144.2 (3 d), 111.0 (2 s, C-5′), 101.7 (d, C-5), 45.2 (t, $NCH_2$), 41.7, 41.6 (2 q, NMe), 37.4 (t, $NCH_2$), 21.4 (2 q, CMe); one C signal in the δ 161–163 region either had insufficient intensity to be detected or overlapped another peak. Anal. Calcd for $C_{18}H_{24}N_8O_{10}Cl_2$: C, 37.06; H, 4.15; N, 19.21. Found: C, 37.08; H, 4.17; N, 19.25.

**Fluorescence Spectra.** Corrected spectra were taken with a Perkin-Elmer MPF-44A instrument. Detection of fluorescence of the most dilute aqueous solutions was faciliated when the correcting unit was not in use. Glass-distilled air-equilibrated water was employed.

**Kinetics of Hydrolysis of 4, 5, and 9.** A few microliters of a $1.4 \times 10^{-3}$ M solution of **4** in acetonitrile was added to 3 mL of 0.01 M borax buffer pH 9.18 at 24 ± 1 °C in a Cary 17D spectrophotometer. The region 440–250 nm was scanned repeatedly, showing an isosbestic point at 290 nm. A first-order plot of the very large absorbance change at 385 nm was liner over 5.0 half-lives with a rate constant of $1.35 \times 10^{-4}$ s$^{-1}$ (half-life 1.4 h). Under the same conditions **5** underwent multiple absorption changes, and the initial isosbestic point at 290 nm became distorted. The absorbance at 250 nm which grew markedly eventually drifted downward somewhat. The first-order plot using the large absorbance change at 385 nm was linear over 4.6 half-lives. The rate constant is $3.55 \times 10^{-4}$ s$^{-1}$ (half-life 32.5 min) for **5**.

In the same borate buffer **9** underwent a change, showing an isosbestic point at about 275 nm. The small absorbance decrease at 250 nm was used to construct a pseudo-first-order plot which was linear over 2.1 half-lives. The rate constant is $3.43 \times 10^{-5}$ s$^{-1}$ (5.62-h half-life).

**Acknowledgment.** The Instrument Program, Chemistry Division of the National Science Foundation, provided financial assistance for the purchase of the Nicolet NT-300 spectrometer used to obtain carbon spectra. Dr. B. Langhammer kindly provided the systematic names. Dr. K. Angelides generously furnished a sample of thiochrome.

**Registry No.** **4**, 86013-89-0; **5**, 86013-93-6; **6**, 86013-95-8; **7**, 86013-97-0; **8**, 86013-99-2; **9**, 86014-01-9; cytidine, 65-46-3; 1′-methylthiaminium diperchlorate, 73333-47-8; 2,4,6-trimethylpyridine, 108-75-8; cytosine, 71-30-7; inosine, 58-63-9; guanosine, 118-00-3; xanthosine, 146-80-5; uracil, 66-22-8.

# Preparation of Optically Pure (*R*)-(+)-Nicotine. Studies on the Microbial Degradation of Nicotinoids

William B. Edwards, III,* and R. McCuen*

*Philip Morris, Inc., Research Center, Richmond, Virginia 23261*

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(*R*)-(+)-Nicotine (**1a**) of high optical purity (average 99.6%) has been obtained from (*R,S*)-nicotine by stereoselective microbial degradation of the (*S*)-(−)-nicotine with use of the microorganism *Pseudomonas putida*. Liquid culture results indicated that this organism growing on (*S*)-(−)-nicotine can utilize **1a** but at a slower rate. Studies on related nicotinoids showed the microorganism to be primarily specific for (*S*)-(−)-nicotine.

## Introduction

Numerous studies on the physiological and insecticidal properties of the optical enantiomers of the alkaloids of *Nicotiana tabacum* L. have been undertaken in an effort to better understand the actions of the naturally occurring alkaloids.[1,2] These investigations have been limited to the

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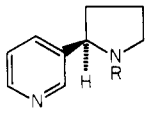
*S*-(−) natural products (*R*)-(+)-nicotine (**1a**) and (*R*)-(+)-nornicotine (**1b**) due to the difficulty in obtaining



**1a**, R = $CH_3$
**b**, R = H

enantiomers of the *Nicotiana* alkaloids. Furthermore, these investigations have, in general, suffered from the lack of a conventient, reliable method for the preparation of optically pure materials.

The most commonly used method for obtaining **1a** has been the repeated fractional crystallization of the (*R,S*)-nicotine *l*-tartrate salt. The **1a** so produced was of varying optical purity (ca. 90–98%).[2a,b] In 1979, Aceto et al. reported a six-step preparation of optically pure **1a** from the racemate using a combination of *d*-tartaric acid and di-*p*-tolouyl-*l*-tartaric acid.[2c] Until quite recently,[3] only Yamashita et al. had investigated the isolation of **1a** from (*R,S*)-nicotine by selective microbial degradation of the (*S*)-(−)-nicotine.[4] This is in strong contrast to considerable work published on the microbial degradation of (*S*)-(−)-nicotine.[5] Yamashita et al. differentiated a *Pseudomonas* type strain[6] into two strains. One of these (the non-6-OH-type microbe) when cultured with (*R,S*)-nicotine oxidized only the (*S*)-(−)-nicotine, leaving nearly pure **1a** (ca. ~99.5%, 60–80% yield). Unfortunately, this non-6-OH-type microbe is no longer available.[7] The alkaloid **1b** has received much less attention than **1a**. Späth et al. isolated optically impure **1b** (ca. 72%) from *Duboisia hopwoodii*.[8] By fractional recrystallization of its diperchlorate salt, **1b** (ca. 98.6%) was obtained.[9] This method has been used with variable success to obtain **1a** (97%)[2d] and (*S*)-(−)-nicotine (ca. 95–100%).[10] As part of our studies[11] on the *Nicotiana* alkaloids, we were interested in examining the physiological consequences of nicotinoid chirality. This article reports our work on the use of the microorganism *Pseudomonas putida* (obtained from Dr. Kisaki of the Japan Tobacco and Salt Public Corp.)[12] to prepare **1a** by selective metabolism of the (*S*)-(−)-isomer in (*R,S*)-nicotine.

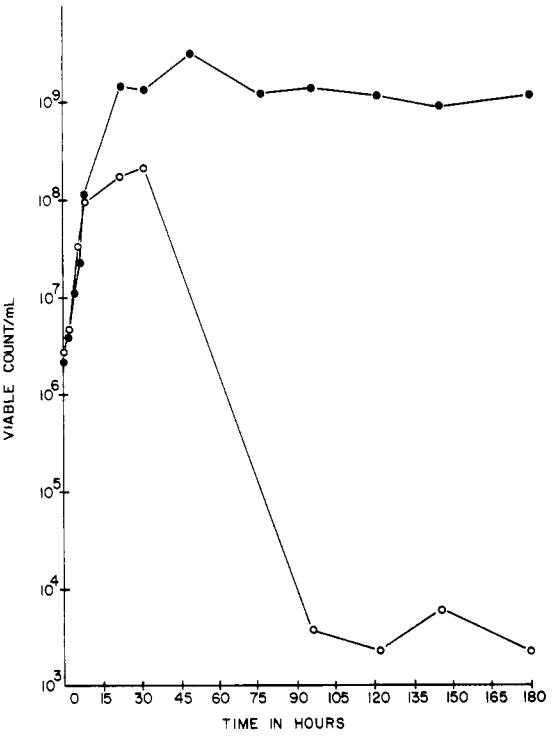



**Figure 1.** Comparison of *P. putida* growth in SM containing 1 mg/mL (solid circles) or 2 mg/mL (open circles) (*S*)-(−)-nicotine. The solid lines show the VC/mL of the culture sampled at various times during the incubation. Each data point is the mean of at least two experiments.

**Table I. Effect of Incubation Time on (*R*)-(+)-Nicotine Recovery**[a]

| time, days | optical rotation,[b] deg | optical purity,[c] % | yield,[d] % |
|---|---|---|---|
| 3 | +166.90 ± 0.33 | 99.12 | 78.5 |
| 5 | +168.18 ± 0.34 | 99.50 | 61.0 |
| 5 | +169.91 ± 0.34 | 100.00 | 75.8 |
| 5 | +167.84 ± 0.34 | 99.40 | 78.4 |
| 5 | +168.70 ± 0.34 | 99.65 | 47.7 |
| 10 | +167.16 ± 0.34 | 99.20 | 23.7 |

[a] Biological incubation conditions and chemical determinations were conducted as described in the Experimental Section. All experiments were conducted with 10 g of (*R,S*)-nicotine. [b] $[\alpha]^{20.0 \pm 0.05}_D$ (neat). [c] Relative to (*S*)-(−)-nicotine $[\alpha]^{20.0 \pm 0.05}_D = -169.92 \pm 0.34°$ (neat). [d] Based on starting **1a** in (*R,S*)-nicotine.

## Results and Discussion

Our initial studies concerned the concentration of (*S*)-(−)-nicotine that gave optimal growth of the organism as determined by viable count (VC) and/or optical density (OD). Figure 1 shows the average results obtained with *P. putida* grown in synthetic medium (SM) containing 1 or 2 mg/mL of (*S*)-(−)-nicotine. Initially (about the first 12 h), the organisms behaved similarly in either nicotine concentration. Upon longer incubation times there was a clear difference. Optimal growth ($1 \times 10^9$ cells/mL) was achieved only when the (*S*)-(−)-nicotine concentration was 1 mg/mL. A concentration of 2 mg/mL of (*S*)-(−)-nicotine exerted a toxic effect on the culture. The OD curves

(1) Secor, H. C.; Edwards, W. B., III *J. Org. Chem.* **1979**, *44*, 3136 and references cited therein. Abood, L. G.; Reynolds, D. T.; Bidlack, J. M. *Life Sci.* **1980**, *27*, 1307. Jenner, P.; Gorrod, J. W.; Beckett, A. H. *Xenobiotica* **1973**, *3*, 573; *Chem. Abstr.* **1974**, *80*, 103739d. Klus, H.; Kuhn, H. *Fachliche Mitt. Austria Tabak A. G.* **1977**, 331; *Chem. Abstr.* **1977**, *87*, 180692e.

(2) (a) Soeda, Y.; Yamamoto, I. *Botyu-Kagaku* **1969**, *34*, 57; *Chem. Abstr.* **1969**, *71*, 80139f. (b) Barlow, R. B.; Hamilton, J. T. *Br. J. Pharmacol.* **1965**, *25*, 206. (c) Aceto, M. D.; et al. *J. Med, Chem.* **1979**, *22*, 174. (d) Hicks, C. S.; Sinclair, D. A. *Aust. J. Exp. Biol. Med. Sci.* **1947**, *25*, 83; *Chem. Abstr.* **1948**, *42*, 675i.

(3) After the completion of this work, a report appeared on the separation of **1a** from (*R,S*)-nicotine by selective degradation of the (*S*)-(−)-nicotine with *Pseudomonas putida*. Detraglia, M. C.; Tometsko, A. M. *Appl. Environ. Microbiol.* **1980**, *39*, 1067. These workers found that in liquid culture their organism could not metabolize **1a** and that its presence did not inhibit degradation of (*S*)-(−)-nicotine by the microbe. Detailed conditions for the preparative production of **1a**, isolated yields for pure **1a**, and error limits for the optical purity of **1a** were not reported.

(4) Yamashita, Y.; Shimizu, S.; Tamaki, E. *J. Agric. Soc. Jpn.* **1963**, *37*, 385; *Chem. Abstr.* **1965**, *63*, 6033d.

(5) Kost, A. N.; Modyanova, L. V. *J. Hetero. Chem. USSR* **1979**, 1049. Geiss, V. L.; et al. U.S. Patent 4 140 136, **1979**; *Chem. Abstr.* **1977**, *87*, 148910p. Gravely, L. E.; Geiss, V. L.; Newton, R. P. U.S. Patent 4 011 141, **1977**; *Chem. Abstr.* **1977**, *86*, 137970m.

(6) Wada, E.; Yamaski, K. *J. Am. Chem. Soc.* **1954**, *76*, 155.

(7) Private communication.

(8) Späth, E.; Hicks, c. S.; Zajic, E. *Ber. Dtsch. Chem. Ges.* **1935**, *68*, 1388.

(9) Späth, E.; Hicks, C. S.; Zajic, E. *Ber. Dtsch. Chem. Ges.* **1936**, *69*, 250.

(10) Kisaki, T.; Tamaki, E. *Arch. Biochem. Biophys.* **1961**, *92*, 351. Späth, E.; Zajic, E. *Ber. Dtsch. Chem. Ges.* **1935**, *68*, 1667.

(11) Pitner, T. P.; Whidby, J. F.; Edwards, W. B., III *J. Am. Chem. Soc.* **1980**, *102*, 5149. Whidby, J. F.; Edwards, W. B., III; Pitner, T. P. *J. Org. Chem.* **1979**, *44*, 794.

(12) The organism was sent to us identified as an *Alcaligenese paradoxus*. It has recently been reclassified as a *Pseudomonas putida*.[7] The similarity/difference between this organism and that reported in ref 3 has not been established.

paralleled the VC data with 1 OD unit being equal to about $1.5 \times 10^9$ viable organisms/mL. The pH curve for cells growing in 1 mg/mL of (*S*)-(−)-nicotine was characterized by a rapid drop from the initial pH of 6.8 to <6.0, followed by a gradual increase to 6.3 as the culture aged.

Because the main emphasis of our studies concerned growing *P. putida* in medium containing (*R,S*)-nicotine, the next studies involved 2 or 4 mg/mL of (*R,S*)-nicotine as the sole carbon source. As expected from previous (*S*)-(−)-nicotine data, the 2 mg/mL (*R,S*)-nicotine concentration produced optimal growth, while the 4 mg/mL concentration exerted toxic effects. The OD and pH data for the lower concentration (2 mg/mL) of racemic nicotine in media were similar to those obtained when only 1 mg/mL of (*S*)-(−)-nicotine was used. Because the data from the racemic mixture experiments were similar to those when only (*S*)-(−)-nicotine was used, it would suggest that (*R*)-(+)-nicotine (**1a**) could not be metabolized by these organisms and that the presence of **1a** did not inhibit the degradation of (*S*)-(−)-nicotine by the microbe. The former finding was consistent with that of Yamashita et al. using a similar biological system.[4]

As a result of these experiments, 2 mg/mL of (*R,S*)-nicotine was employed to determine the optimal incubation time necessary to remove (*S*)-(−)-nicotine from the culture medium. Cultures were incubated for 3, 5, or 10 days after which the nicotine concentration remaining in the cell-free culture medium was determined and its optical purity was measured. The results of this experiment along with three replicate experiments conducted at an incubation time of 5 days are presented in Table I. These data showed that under these conditions, 5 days of incubation were optimum for producing optically pure **1a** although the yields were somewhat variable. Our interpretation of the observed decrease in yield of **1a** with increased incubation time and the failure to obtain near-quantitative yields is that *P. putida* was metabolizing **1a**.

Due to an interest in other alkaloids, particularly those related to (*S*)-(−)-nicotine, the ability of *P. putida* to use the following compounds was examined: 3-(3,4-dihydro-2*H*-pyrrol-5-yl)pyridine (myosmine), (*S*)-(−)-1-methyl-2-(3-pyridyl)piperidine ((*S*)-(−)-*N*′-methylanabasine), (*S*)-(−)-2-(3-pyridyl)piperidine ((*S*)-(−)-anabasine), 1-methyl-2-(2-pyridyl)pyrrolidine (2-nicotine), (*S*)-(−)-1-methyl-5-(3-pyridyl)-2-pyrrolidinone ((*S*)-(−)-cotinine), 2-(3-pyridyl)pyrrolidine (nornicotine), and 1-methyl-2-phenylpyrrolidine. The study was conducted with agar plates as there were insufficient quantities of the alkaloids of interest for liquid-culture tests. Minute quantities of selected nicotinoids were applied to the surface of agar plates previously seeded with a high concentration of starved log phase *P. putida* cells. After a suitable incubation period, the plates were examined for growth in the vicinity of the test compounds. While it was clear that all of the compounds were able to permeate the cells, the organisms were able to grow on medium only containing the control alkaloids: (*S*)-(−)- or (*R,S*)-nicotine. The agar plate data on **1a** and (*R,S*)-nicotine, while in agreement with the conclusions obtained by Yamashita et al.,[4] was inconsistent with out interpretation of the results (Table I) that indicated that **1a** was being utilized in liquid-culture medium.

It was unclear based on our studies up to this point whether the culture conditions (liquid vs. agar plate) played a role in the degradation of **1a** by *P. putida*. Consequently, starved log phase cells ($1 \times 10^4$/mL) were incubated in SM containing **1a** (0.5 mg/mL, optical purity 99.56%) and as controls (*S*)-(−)-nicotine (2.2 μg/mL, level of residual (*S*)-(−)-nicotine in **1a**), (*S*)-(−)-nicotine (0.5 mg/mL), or no nicotine. Over 7 days, significant growth was observed only in the system initially containing (*S*)-(−)-nicotine at 0.5 mg/mL. It was, therefore, concluded that the culture conditions did not appreciably affect the utilization of **1a** by *P. putida*.

In conclusion, this paper describes a detailed method for the production of large quantities of **1a** of high optical purity (av 99.6%). Agar plate results suggested that *P. putida* could not metabolize **1a** or other nicotinoids. Liquid-culture studies and yield data indicated that *P. putida* growning on (*S*)-(−)-nicotine can utilize **1a** but at a slower rate.

## Experimental Section

Boiling points are uncorrected. The $^1$H NMR spectra were determined on a Bruker WP-80 spectrometer operating in the pulse-FT mode with $Me_4Si$ as an internal standard. GLC and preparative GLC (PGLC) were carried out with a Bendix Model 2300 chromatograph with 1.5 m × 0.64 cm stainless steel columns packed with 5% SE-30 on Chromosorb G-HP (80–100 mesh) with He carrier gas at 60 mL/min flow rate, using a TC detector or a Bendix Model 2200 chromatograph with 18.3 m × 0.32 cm copper column packed with 3% C20M on SD (70–80 mesh) with He carrier gas at 25 mL/min flow rate, using a flame-ionization detector. Optical rotations were determined on a Perkin-Elmer 241MC polarimeter equipped with a Neslab RTE-4 refrigerated bath circulator and using a quartz short-path microcell of 10-mm length and 0.1-mL volume. Optical density (OD) determinations were done at a wavelength of 660 μm, using a double-beam, Perkin-Elmer 124D spectrophotometer. TLC was run on Analtech 250-μm $SiO_2$ GF plates with $CHCl_3$/MeOH/$NH_4OH$ (85:14:1) as the eluting solvent. Preparative TLC (PTLC) was run on Analtech 1000-μm $SiO_2$ GF plates with the same eluting solvent.

**Reagents.** (*S*)-(−)-Nicotine (Kodak Laboratory Chemicals) was distilled under reduced pressure. The (*S*)-(−)-nicotine boiling at 55–56 °C (0.03 mmHg) was collected and stored in full sealed brown glass bottles under $N_2$. TLC and GLC showed a purity of essentially 100%. Optical rotation was determined as $[\alpha]^{20.0\pm0.05}_D = -169.92 \pm 0.34°$ (neat). (*R,S*)-Nicotine was prepared by using established procedures:[13] reduction of myosmine with $NaBH_4$ to nornicotine and its methylation with $CH_2O$/$HCO_2H$. Purification was carried out as with (*S*)-(−)-nicotine to give (*R,S*)-nicotine, which TLC and GLC showed to be essentially 100% pure. The 2-propanol used was from Burdick and Jackson Laboratories Inc.

**Microorganisms.** The organism used in these studies was kindly supplied to us by Dr. Kisaki of the Japan Tobacco and Salt Public Corp. The bacterium was initially determined to be *Arthrobacter globiformis*, which was then reclassified as an *Alcaligenes paradoxus* JTS0003 (formerly strain Nic-64). Quite recently the microorganism was reidentified as *Pseudomonas putida*. It is filed with the Fermentation Research Institute Agency of Industrial Science and Technology in Japan as FERM P-6146. It is also stocked by the Japan Tobacco and Salt Public Corp. as JTS-9.

**Maintenance of the Cells.** The cells were routinely grown in a completely synthetic medium that contained the following chemicals (amount/liter): 1.5 g of $KH_2PO_4$, 0.5 g of $MgSO_4$, 0.003 g of $FeSO_4$, 0.003 g of $CaCl_2$, 0.003 g of $MnSO_4$ (SM), and various concentrations of nicotine as described below. The pH was adjusted to 6.8 with 10 N HCl. The medium was filter sterilized (Nalgene filter, 0.2-μm pore size), dispersed into screw-cap containers, and stored at 4 °C in the dark until used. For producing agar slants that were used to make stock cultures, 30 g of Bacto agar (Difco) was added to 1 L of SM containing 1 g of (*S*)-(−)-nicotine. Viable counts (VC) were conducted by making serial, 10-fold dilutions of the organism in SM and spreading 0.1 mL of nutrient agar (Difco) plates. The plates were incubated for 24 h at 30 °C and the resulting colonies were counted with a Quebec Colony Counter. Stock cultures were prepared from single-colony isolates grown on agar slants. These were stored

(13) Glenn, D. F.; Edwards, W. B., III *J. Org. Chem.* **1978**, *43*, 2860.

at 4 °C and after 6 months use were discarded and new stock cultures were prepared.

**Microbial Degradation of Nicotine in Liquid Cultures.** The following procedure was employed unless stated otherwise. One loop of organisms from the stock culture slant was inoculated into 125 mL of SM containing 1 mg/mL of (*S*)-(−)-nicotine in a 250-mL Erlenmeyer screw-cap flask. The top was loosely taped on and the flask incubated at 30 °C in a G-76 gyrotary shaker (New Brunswick Scientific) at 150 rpm. (Cultures were usually grown in Erlenmeyer flasks filled to one-half of their volume with media.) After a 48-h incubation period, the flask containing late log-phase cells was sampled for a VC and OD determination. On the basis of the OD value, the cells were diluted in 500 mL of SM containing the desired concentration of nicotine in a 1-L Erlenmeyer flask so that the final concentration of cells gave an OD reading of 0.001, which was equivalent to $\sim 1.5 \times 10^6$ cells/mL. The cultures were incubated as described for the desired length of time after which the cells were removed from the medium by low-speed centrifugation (5000 g, 5 °C, 10 min), followed by filtration of the supernatant solution (Nalgene filter, 0.2-$\mu$m pore size) and storage of the filtrate at 4 °C in glass, screw-cap bottles. These solutions were used for nicotine isolation (see below).

The liquid culture experiments involving different concentrations of (*R*)-(+)- or (*S*)-(−)-nicotine or no nicotine were conducted with use of starved *P. putida* cells. Log-phase cells ($5 \times 10^8$/mL) growing in SM supplemented with 1 mg/mL (*S*)-(−)-nicotine were starved for 6 h by incubating saline-washed cells in SM. Starved cells were washed twice with sterile saline and resuspended in saline at a concentrations of $1 \times 10^9$ cells/mL. These cells were diluted in saline and used as stated in the Results and Discussion section.

**Microbial Degradation of Alkaloids on Agar Plates.** Starved log-phase cells were produced as described above. For making agar plates, 30 g of Bacto agar (Difco) was added to 1 L of SM. These agar plates were spread with 0.1 mL of the undiluted ($10^9$ cells/mL), washed, starved cells. After the inoculated plates were dried for 5 min at room temperature, 1 drop or a few crystals of each of the test compounds was placed near the edge of each plate. In addition to the test compound, each plate contained 1 drop of each of the control compounds ((*S*)-(−)-nicotine and (*R*,*S*)-nicotine). The plates were incubated for 2 weeks at 30 °C and observed daily for toxicity (a clear area surrounding each of the compounds, which indicated that the compound was able to permeate the cells) and growth (a 1–3-cm turbid area lying just outside the clear toxicity area).

**Nicotine Isolation.** The cell-free nicotine-containing reaction solution from a 5-day microbial degradation of 5 L of medium with an initial (*R*,*S*)-nicotine concentration of 2 mg/mL was acidified to pH 2 with 10% HCl (ca. 200 mL). The solution was concentrated under reduced pressure to ca. 250 mL. The concentrate was cooled (0–5 °C) and basified with excess 50% NaOH (pH 11, ca. 50 mL). A solid separated, which was removed by filtration and washed with $3 \times 25$ mL 10% NaOH and $4 \times 50$ mL of $Et_2O$. The aqueous filtrate and washings were combined and extracted continuously with $Et_2O$ for 48 h. The combined $Et_2O$ washings and $Et_2O$ extract were dried (NaOH), and the $Et_2O$ was removed [20 °C (15 mmHg)] to leave 4.03 g of an oil. Control experiments using 20.62 mg of (*S*)-(−)-nicotine in 990 mL of medium and 89.1 mg in 175 mL of medium showed 91% recovery of (*S*)-(−)-nicotine, using this procedure.

**(*R*)-(+)-Nicotine (1a) Purification.** The isolated crude product from all experiments showed only nicotine and trace solvent by GLC. TLC of these crude products showed them to be primarily nicotine ($R_f$ 0.62) with varying trace contaminants at $R_f$ 0.05, 0.10, and 0.40 as visualized by Dragendorff reagent. Crude product (3.33 g) from a 5-day microbial degradation of 10 g of (*R*,*S*)-nicotine that showed trace impurities at $R_f$ 0.05 and 0.10 by TLC was distilled in vacuo through a 5-cm Vigreaux column. The product boiling from 39 to 44 °C (0.025 mmHg) was collected (3.05 g). GLC showed only nicotine, while the TLC was unchanged. The optical rotation was $[\alpha]^{20.0\pm0.05}_D = +168.18 \pm 0.34°$ (neat). Redistillation gave 2.96 g with the same GLC and TLC and $[\alpha]^{20.0\pm0.05}_D = +168.14 \pm 0.34°$ (neat). PTLC was run on the product. The product band was collected, placed in a Soxhlet extraction apparatus, and continuously extracted with glyme (freshly dried over $LiAlH_4$) for 40 h under $N_2$. The glyme was cooled (5 °C), treated with 20 mL of 10% HCl, and removed under reduced pressure. The remaining aqueous acid was cooled (0 °C), basified with excess 50% NaOH (pH 11), and extracted with $Et_2O$. The $Et_2O$ was dried (NaOH) and removed [20 °C (15 mmHg)] to give 2.75 g of an oil. The oil was distilled as previously described to give 2.48 g of **1a**: bp 58–60 °C (0.35 mmHg); $[\alpha]^{20.0\pm0.05}_D = +168.43 \pm 0.34°$ (neat). TLC, GLC, and NMR showed only nicotine.

**Determination of Nicotine in Media.** The general GLC procedure of Randolph[14] was used to determine the nicotine concentration in media. For controls, the nicotine-containing medium was used directly, while for experimental runs the cell-free extract was employed. For nicotine concentrations ranging from 8 to 0.5 mg/mL, 2.50 mL of nicotine-containing medium was added to 10.0 mL of 2-propanol in a 15 × 125 mm test tube. The cloudy solution was placed under $N_2$ and the test tube capped with a serum stopper. These samples were run against standards in 2-propanol. Control experiments showed that the approximately 20% $H_2O$ in the samples did not affect the accuracy of the determination. For nicotine concentrations from about 0.5 mg/mL to <0.05 mg/mL, 0.5 mL of nicotine-containing media was placed in a 10 × 75 mm test tube under $N_2$ and the test tube was capped with a serum stopper. These samples were run against standards in media at 0.5, 0.4, 0.3, 0.2, 0.1, and 0.05 mg of nicotine/mL. Control experiments showed that this method did not affect the accuracy of the determination.

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**Registry No.** **1a**, 25162-00-9; (*R*,*S*)-nicotine, 22083-74-5; (*S*)-(−)-nicotine, 54-11-5.

(14) Randolph, H. R. *Tob. Sci.* **1974**, *18*, 137; *Chem. Abstr.* **1975**, *82*, 95460e.