Microbial Transformations of Natural Antitumor Agents XXII: Conversion of Bouvardin to *O*-Desmethylbouvardin and Bouvardin Catechol

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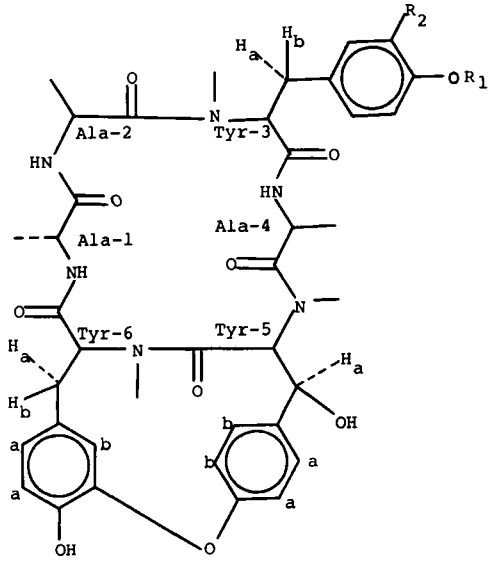
Abstract □ Bouvardin is a cyclic hexapeptide antitumor agent which undergoes two major microbial transformation reactions. Screening with 220 cultures revealed 17 different strains capable of producing *O*-desmethylbouvardin in good yield. *O*-Desmethylbouvardin was isolated and characterized from preparative scale incubations with *Streptomyces rutgersensis* NRRL B-1256. Four aspergilli and one streptomycete formed bouvardin catechol when *O*-desmethylbouvardin was used as substrate. Bouvardin catechol was isolated and characterized from a preparative scale incubation with *Aspergillus ochraceous* UI 398.

Keyphrases □ Bouvardin—conversion to *O*-desmethylbouvardin and bouvardin catechol, microbial transformation, *Streptomyces rutgersensis, Aspergillus ochraceous* □ Microbial transformation—bouvardin to *O*-desmethylbouvardin and bouvardin catechol, *Streptomyces rutgersensis, Aspergillus ochraceous* □ Metabolites—bouvardin, microbial transformation to *O*-desmethylbouvardin and bouvardin catechol, *Streptomyces rutgersensis, Aspergillus ochraceous*

Bouvardin (I), a novel cyclic hexapeptide antitumor agent isolated from the plant *Bouvardia ternifolia* (Rubiaceae) (1), has demonstrated antitumor activity against the P-388 lymphocytic leukemia test system and against B-16 melanoma (1). Modification of bouvardin by synthetic methods appeared to be difficult; thus, this material was deemed an excellent candidate for microbiological modification (2). This report describes microbial transformation experiments of bouvardin which afforded two major metabolites, *O*-desmethylbouvardin (II) and bouvardin catechol (III).



I: $R_1 = CH_3$, $R_2 = H$
II: $R_1 = R_2 = H$
III: $R_1 = H$, $R_2 = OH$

EXPERIMENTAL

Melting points were determined in open-ended capillary tubes and are corrected. UV spectra[1] were measured in ethanol solutions, and IR spectra[2] were determined in potassium bromide pellets. ^{1}H-NMR spectra[3] were obtained in $CDCl_3$ using tetramethylsilane as an internal standard. ^{13}C-NMR spectra were obtained in DMSO-d_6 at 90 MHz with an instrument operating at 22.635 MHz. Assignments for appropriate carbon signals were confirmed by use of off-resonance noise decoupling and delayed decoupling techniques to indicate multiplicities of carbons due to carbon-proton splittings. Low-resolution mass spectra[4] were obtained using a direct-inlet probe, and high-resolution and field-desorption mass spectra were provided through the mass spectral facility of the Massachusetts Institute of Technology, Cambridge, Mass.

Chromatography—TLC was performed on buffered 0.25-mm layers of silica gel GF_{254} on glass plates. Plates were prepared by spreading a slurry of 25 g of silica gel GF_{254} in 50 ml of 4% aqueous KH_2PO_4. After air drying, plates were uniformly deactivated by predevelopment with methyl ethyl ketone saturated with water. Plates were developed with methylene chloride–methanol–water (90:10:0.5 or 85:10:0.5), and developed chromatograms were visualized by fluorescence quenching under 254-nm UV light, or by spraying with Dragendorff's reagent (3) or with ceric ammonium sulfate reagent [1% $Ce(NH_4)_4(SO_4)_4$ in 50% H_3PO_4] and warming the sprayed plates with a heat gun.

Bouvardin (I)—Bouvardin was obtained[5] as a crystalline compound which possessed the following physical properties: mp 240–241°; UV (EtOH): 278 (ϵ 3780) and 284 nm (3370); IR: 3620 (OH phenolic), 1680 (amide C=O), and 1245 cm^{-1} (C—O—C); ^{1}H-NMR: see Table I; mass spectrum (field-desorption): m/z 772 for $C_{40}H_{48}N_6O_{10}$; high-resolution mass spectral fragments obtained by electron-impact spectroscopy: see Table II. ^{13}C-NMR spectral data was obtained on a DMSO-d_6 solution of bouvardin and was identical to that reported by Bates *et al.* (4).

Fermentation Procedure—All cultures are maintained in the University of Iowa, College of Pharmacy culture collection. Cultures were grown according to the usual two-stage fermentation procedure used in these laboratories (5) in a soybean meal–glucose medium of the following composition: 5 g of soybean meal; 20 g of glucose, 5 g of yeast extract, 5 g of sodium chloride, 5 g of dibasic potassium phosphate, and 1000 ml of distilled water; the pH was adjusted to 7.0 with 6 *N* HCl. Nutrient broth[6] was employed in the cultivation of bacterial strains. Media were sterilized in an autoclave at 121° for 15 min prior to use.

Fermentations were conducted on rotary shakers[7] operating at 250 rpm and describing a 2.5-cm orbital stroke at 27° in steel-capped Delong culture flasks containing one-fifth of their volumes of culture medium. Fermentations were initiated by suspending the surface growth from slants in 5 ml of sterile medium and by using the resulting suspension to inoculate stage I cultures. Thick 72-hr stage I cultures were used to inoculate stage II fermentations; the inoculum volume consisted of 10% of the volume of medium held in the stage II culture flasks. Bouvardin was added as substrate to 24-hr stage II cultures in a dimethylformamide solution, and the progress of microbial transformation reactions was monitored by TLC. For this, 4-ml samples were withdrawn at various time intervals, adjusted to pH 6.5 by the addition of 1 ml of 2 *M* pH 6.3

[1] Model SP1800; Pye Unicam Ltd., Cambridge, England.
[2] Perkin-Elmer Model 267, Norwalk, Conn.
[3] Bruker Model WM250 or Bruker FX90Q, USA Bruker Instruments, Inc., Billerica, MA 01821.
[4] Model 3200; Finnigan Corp., Sunnyvale Calif.
[5] National Cancer Institute, DTP, DCT, Silver Spring, MD 20910.
[6] Difco Laboratories, Detroit, Mich.
[7] Model G-25; New Brunswick Scientific Co., Edison, N.J.

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Table I—^{1}H-NMR [a] Chemical Shifts (δ) and Coupling Constants (Hz) for Bouvardin (I), *O*-Desmethylbouvardin (II), and Bouvardin Catechol (III) in $CDCl_3$

	I	II	III
Ala-4β	1.08d (6.6)	1.10	1.09
Ala-1β	1.30d (7.0)	1.30	1.31
Ala-2β	1.37d (6.9)	1.37	1.37
Tyr-6-N-Me	2.74s	2.74	2.74
Tyr-3-N-Me	2.87s	2.88	2.91
Tyr-6β_a	2.91dd (18.8, 3)	2.90	2.90
Tyr-6β_b	3.12dd (18.8, 10.8)	3.11	3.12
Tyr-5-N-Me	3.33s	3.33	3.33
Tyr-3β	3.35m	3.35m	~3.31m
Tyr-3α	3.62dd (9.2, 5.9)	3.61	3.62
Tyr-3-O-Me	3.80s	—	—
Tyr-6δ_b	4.35d (1.8)	4.35	4.36
Tyr-6α	4.37m	4.33	4.34
Ala-1α	4.38 ~ p (7)	4.37	4.38
Ala-2α	4.76 ~ p (7)	4.77	4.77
Ala-4α	4.90 ~ p (7)	4.89	4.89
Tyr-5β_a	5.08dd (10.2, 1.8)	5.07	5.07
Tyr-5α	5.36d (1.8)	5.36	5.37
Tyr-6-OH	5.58s	5.61	5.79
Ala-1-NH	6.00d (7.1)	6.05	6.16
Ala-4-NH	6.44d (7.7)	6.44	6.47
Tyr-5-OH	6.50d (10.2)	6.51	6.52
Tyr-6δ_a	6.51dd (8.1, 1.8)	6.53	6.54
Ala-2-NH	6.65d (7.9)	6.66	6.68
Tyr-3ϵ	6.81 ~ d (8.7)	6.78	6.81d (7.4)
Tyr-6ϵ_a	6.84d (8.1)	6.84	6.84
Tyr-5ϵ_b	6.95dd (8.7, 2.2)	6.97	6.97
Tyr-3δ	7.05 ~ d (8.5)	7.01	6.53dd (7.4, 2.0)
Tyr-5ϵ_a	7.23dd (8.5, 2.2)	7.25	7.25
Tyr-5δ_b	7.38dd (8.5, 2.2)	7.39	7.39
Tyr-5δ_a	7.50dd (8.7, 2.2)	7.51	7.51
Tyr-3-OH	—	4.88s	6.40, 6.43s

[a] 250 MHz.

Table II—High-Resolution Electron-Impact Mass Spectral Fragments Obtained With Bouvardin (I), *O*-Desmethylbouvardin (II), and Bouvardin Catechol (III)

Empirical Formula	Calculated Mass	Observed Mass (Percent Relative Abundance)		
		I	II	III
C_6H_6O	94.04186	94.04336 (11.91)	94.04286 (87.83)	94.04137(51.29)
C_7H_7O	107.04969	107.04907 (17.44)	107.04833 (100)	107.04845 (56.71)
$C_6H_6O_2$	110.03678	—	110.03544 (16.61)	110.03977 (80.49)
$C_5H_8N_2O$	112.06366	112.06352 (21.23)	112.06363 (55.39)	112.06323 (61.88)
$C_7H_7O_2$	123.04460	—	123.04436 (5.86)	123.04994 (100)
$C_7H_{10}N_2O$	138.07931	138.07939 (27.92)	138.07909 (66.82)	138.08114 (78.14)
C_8H_9O	121.06534	121.06343 (100)	121.06391 (45.05)	—
$C_{14}H_{13}O_2$	213.09155	213.09087 (1.0)	213.09246 (73.50)	213.09072 (1.4)

phosphate buffer, and extracted with 1 ml of ethyl acetate. Approximately 30 μl of the extracts were spotted onto TLC plates for analysis.

Small-scale fermentations were used to screen 220 microorganisms for their abilities to metabolize bouvardin. Screening experiments were conducted in 125-ml steel-capped Delong flasks in 25 ml of medium, and 10 mg of bouvardin was added to each culture. Initial screening results were confirmed in a second experiment with control cultures containing no substrate and with autoclaved cultures incubated with bouvardin.

Preparation of *O*-Desmethylbouvardin (II) from Bouvardin by *Streptomyces rutgersensis* (NRRL) B-1256)—*S. rutgersensis* was grown in 500- or 1000-ml culture flasks, and a total of 3.36 g of I in 22.4 ml of dimethylformamide was evenly dispensed to a final concentration of 0.6 mg of bouvardin/ml of culture medium in the 24-hr stage II cultures. Conversions of bouvardin to one major metabolite commenced within 24 hr and neared completion after 12 days of incubation (TLC). Cells were separated from the fermentation beer by filtration, and the combined filtrates were adjusted to pH 6.5 with 10% aqueous KOH. The filtrate (5.5 liters) was extracted exhaustively with ethyl acetate (2 × 2.5 liters) and then with ethyl acetate–2-propanol (9:1, 2 × 2 liters). The extracts were combined, dried over anhydrous sodium sulfate, filtered, and evaporated to a thick oily residue. This residue was triturated with water to remove the dimethylformamide, and the resulting suspension was filtered through a sintered glass funnel to collect the bouvardin-containing solids. The crude product (4.57 g) was purified by column chromatography on silica gel[8] (90 g, 3 × 35 cm) using a solvent system of methylene chloride–methanol–water (90:10:0.5) at a flow rate of 0.7 ml/min; 20-ml fractions were collected. Fractions 17–24 contained bouvardin (0.49 g), while fractions 25–42 provided *O*-desmethylbouvardin (II) (2.53 g, 79% yield). Recrystallization of the metabolite from ethyl acetate yielded the analytical sample (1.91 g): mp 224.5–227.0°; UV: 239.5 (6850), 278 (3540), and 280 nm (3560); IR: 3380 (OH phenolic), 1680 (amide C=O), and 1245 cm^{-1} (C—O—C); ^{1}H-NMR; see Table I; mass spectrum (field-desorption): *m/z* 797 ($M + K^+$), 781 ($M + Na^+$), 758 (M^+ for $C_{39}H_{46}N_6O_{10}$), and 740 ($M - H_2O$); electron-impact mass spectral data: see Table II.

Preparation of Bouvardin Catechol (III) from *O*-Desmethylbouvardin (II) by *Aspergillus ochraceous*—*A. ochraceous* (UI 398) was grown in 12, 1-liter Delong culture flasks for the stage II incubation. A total of 960 mg of II was dissolved in 9.6 ml of dimethylformamide and dispensed evenly among the cultures to give a final *O*-desmethylbouvardin concentration of 0.4 mg/ml in culture medium. Ascorbic acid (500 mg in 4 ml of H_2O) was added to each flask at 48 hr after addition of *O*-desmethylbouvardin, when TLC analyses first indicated the presence of III (6). After 96 hr, TLC estimation indicated an 80% conversion of II to III, and the fermentation was harvested.

Cells were separated from the medium by filtration through cheesecloth, and the cells were extracted with ethyl acetate (200 ml). This extract was later combined with culture filtrate extracts. The filtrate (2000 ml) was extracted with ethyl acetate (3 × 1 liter) and then with ethyl acetate–2-propanol (9:1, 1 liter). After drying the combined extracts over anhydrous sodium sulfate, evaporation yielded an oily residue (800 mg), which was dissolved in methylene chloride–methanol–water (90:10:0.5, 5 ml) and applied to silica gel column (90 g, 3 × 35 cm). The column was

[8] Baker 3405; J. T. Baker Chemical Co., Phillipsburg, N.J.

eluted at a flow rate of 0.7 ml/min while 20-ml fractions were collected. After 50 fractions, the solvent was changed to methylene chloride–methanol–water (90:20:0.5) for an additional 30 fractions. Fractions 14–24 contained unreacted *O*-desmethylbouvardin (II) (164 mg), fractions 25 and 26 contained a mixture of II and III (49 mg), and fractions 27–55 contained bouvardin catechol (III) (280 mg, 34.5% yield based on substrate utilized). The crude bouvardin catechol was dissolved in methyl ethyl ketone (10 ml), and 50 ml of hexane was added to precipitate 262 mg of the catechol (III) from solution. This material was collected by filtration and gave the following physical data: mp 221–223°; UV: 284 nm (2740); IR: 3350 (broad, OH phenolic), 1650 (amide C=O), and 1270 cm^{-1} (C—O—C); ^{1}H-NMR: see Table I; mass spectrum (field-desorption): *m/z* 797 ($M + Na^+$) and 774 (M^+ for $C_{39}H_{46}N_6O_{11}$); electron-impact spectrum: see Table II. The Arnow's test was positive, indicative of a catechol moiety in the structure of the metabolite (7).

RESULTS AND DISCUSSION

Compounds such as bouvardin whose structural complexity render total synthesis or chemical modification difficult are excellent substrates for microbiological modification (2). The present study was designed to provide metabolites of bouvardin in sufficient quantity for biological evaluation to extend the structure–activity relationships of the antitumor peptide. Small-scale screening experiments with 220 cultures resulted in the identification of 17 microorganisms which accumulated the same major metabolite (Table III). The metabolite was produced in consistently high yield (TLC) by *S. rutgersensis* (NRRL B-1256), and this organism was selected for preparative-scale fermentation to obtain sufficient amounts of the metabolite for structure elucidation and biological testing. The metabolite was obtained in 79% yield in the preparative-scale incubation, and ^{1}H-NMR and mass spectral measurements supported its structure as *O*-desmethylbouvardin (II).

The ^{1}H-NMR spectrum of the metabolite was identical to that of bouvardin, except for the absence of the signal for the methoxyl group protons of the tyrosine-3 residue (Table I) and the presence of a new hydroxyl signal at 4.48 ppm. Similarly, the signal for the methoxyl carbon atom of bouvardin, which resonates at 55.494 ppm, was absent in the ^{13}C-NMR spectrum of the metabolite. Field-desorption mass spectral measurement provided a molecular ion of *m/z* 758 for the metabolite *versus m/z* 772 for bouvardin. While the electron-impact mass spectrum failed to provide ions at higher mass, the base peak of the metabolite spectrum was at *m/z* 107.04833 for C_7H_7O (Table II). This peak is derived from the *N*-methyltyrosine-3 residue of the metabolite. In bouvardin, the base peak exists at *m/z* 121.06343 for C_8H_9O, the methoxylated aromatic ring derived from the tyrosine-3 amino acid residue. These data strongly support the structure of the major bouvardin metabolite as *O*-desmethylbouvardin (II).

Since II could be efficiently produced by microbial transformation of bouvardin, this metabolite was also utilized as a substrate in microbial transformation experiments. *O*-Desmethylbouvardin (II) was screened with 10 cultures known to accomplish aromatic hydroxylation. Of the cultures examined, *Aspergillus alliaceus* (NRRL 315), *A. ochraceous* (UI 398). *A. ochraceous* (NRRL 1008), and *Streptomyces griseus* (UI 1158) provided the same more polar (TLC) metabolite of II. *A. ochraceous* UI 398 gave the highest and most consistent yields of the new metabolite, and it was used in a preparative-scale incubation. In the preparative-scale fermentation, ascorbic acid was employed to prevent possible degradation of the phenolic starting material and the presumed catechol metabolite that was being formed. A similar approach was utilized in the microbial oxidation of *N*-blocked tyrosine residues to form *N*-blocked levodopa derivatives (6). Following solvent extraction and chromatography, the metabolite was obtained in 34.5% yield and was identified as bouvardin catechol by spectral and chemical methods.

A positive Arnow's test suggested that the metabolite possessed a catechol moiety in its structure (7). While the metabolite gave a positive test, *O*-desmethylbouvardin and bouvardin were negative. Field-desorption mass spectrometry indicated a molecular weight of 774 for the metabolite, consistent with the addition of a single oxygen atom to *O*-desmethylbouvardin. The base peak in the electron-impact spectrum (Table II) of the metabolite was at *m/z* 123.04461 for $C_7H_7O_2$, which would be expected if an oxygen atom had been introduced into the tyrosine-3 residue of *O*-desmethylbouvardin. This fragment is supported by the presence of a characteristic catechol fragment at *m/z* 110.03977 for $C_6H_6O_2$, which was intense in the spectrum of the metabolite but relatively insignificant in the spectrum of *O*-desmethylbouvardin or bouvardin (Table II).

A detailed analysis of the ^{1}H- and ^{13}C-NMR spectral properties of bouvardin has been made (4); this provided a useful tool in establishing the structure of the new bouvardin metabolite. The 250-MHz ^{1}H-NMR spectrum of the metabolite strongly supported its proposed structure as bouvardin catechol (III). All of the resonances of the metabolite were virtually identical for I, II, and III except for signals attributed to the aromatic protons of the tyrosine-3 moiety and for OH and NH absorptions which moved farther downfield in the spectrum of III due to concentration differences. The tyrosine-3 absorption changed in the expected ways for III. The aromatic absorption pattern for this metabolite resembled that of 4-methyl catechol (8) as a typical ABX system with protons for the Tyr-3δ [6.53 ppm (dd, J = 7.4 and 2.0 Hz) and 6.70 ppm (d, J = 2.0 Hz)] and Tyr-3ϵ [6.81 ppm (d, J = 7.4 Hz)] possessing the appropriate coupling constants. These data confirm the structure of the metabolite as that of bouvardin catechol (III).

Table III—Cultures[a] Producing *O*-Desmethylbouvardin (II) from Bouvardin (I).

Corynebacterium mediolarum (ATCC 14004)
Cyathus striatus (UI 356)
Gelasinospora autosteria (UIGA)
Hansenula species (UI-HCY)
Helicostylum piriforme (QM 6944)
Lentodium squamulosum (UI 1566)
Mycobacterium fortuitum (UI 53378)
Nannizzia cajetana (−) (UI 1128)
Phanerochaete chrysosporium (UI 446)
Poria monticola (UI 332)
Pseudomonas putida (ATCC 17453)
Sepedonium chrysospermum (ATCC 13378)
Sordaria bomboidee (UI 183)
Streptomyces rimosus (NRRL 2234)
S. rimosus (ATCC 14673)
Streptomyces rutgersensis (NRRL B-1256)
Unidentified Yeast (UI 1477)

[a] ATCC, American Type Culture Collection, Rockville, Md.; NRRL, Northern Regional Research Laboratories, Peoria, Ill. UI, University of Iowa, College of Pharmacy Culture Collection, Iowa City, Iowa; QM Quartermaster Culture Collection, U.S. Army Laboratories, Natick, Mass.

Additional attempts were made to modify bouvardin by employing cultures known to form peptide antibiotics containing α,β-unsaturation or dehydro-amino acid moieties in their structural backbones. It was reasoned that formation of the dehydro-moieties could occur as late biosynthetic reactions in the formation of antibiotics such as mikamycin (9), subtilin (10), dehydro-*N*-benzyloxycarbonyl-L-tryptophan (11), glycocinnamoyl-spermidines (12), antibiotic A32390A (13), telomycin (14), stendomycin (15), trichostatin C (16), and ostreogrycin (17), and that the introduction of this type of functionality into bouvardin could result in enhanced or novel antitumor activity. Cultures were grown in nutrient broth with and without supplements of phenylalanine, alanine, or tyrosine as possible inducers of enzymes capable of dehydrogenating amino acids (11). None of the antibiotic-producing cultures formed novel derivatives of bouvardin. In these experiments, *Nocardia* species (NRRL 5646), *Streptomyces hygroscopicus* (ATCC 32431), and *Actinoplanes philippinensis* (NRRL 5462) formed *O*-desmethylbouvardin from bouvardin, albeit in lower yields than *S. rutgersensis*.

Bouvardin and its metabolites, *O*-desmethylbouvardin (II) and bouvardin catechol (III) were submitted to the National Cancer Institute for biological testing *versus* the P-388 leukemia test system. None of the metabolites were active, thus indicating that the *O*-desmethylation and further hydroxylation reactions are bioinactivation processes.

REFERENCES

(1) S. D. Jolad, J. J. Hoffmann, S. J. Torrance, R. M. Wiedhopf, J. R. Cole, S. K. Arora, R. B. Bates, R. L. Gargiulo, and G. R. Kriek, *J. Am. Chem. Soc.*, **99**, 9040 (1977).

(2) J. P. Rosazza, in "Anticancer Agents Based on Natural Product Models," J. Cassady and J. Douros, Eds., Academic, New York, N.Y., 1981, pp. 437–463.

(3) E. Stahl, "Thin Layer Chromatography," 2nd ed., Springer-Verlag, New York, N.Y., 1969, p. 873 (No. 94, only solution A was used).

(4) R. B. Bates, J. R. Cole, J. R. Hoffmann, G. R. Kreik, G. S. Linz, and S. J. Torrance *J. Am. Chem. Soc.*, in press.

(5) R. E. Betts, D. E. Walters, and J. P. Rosazza, *J. Med. Chem.*, **17**, 599 (1975).

(6) C. J. Sih, P. Foss, J. Rosazza, and M. Lemberger, *J. Am. Chem. Soc.*, **91**, 6204 (1969).

(7) L. E. Arnow, *J. Biol. Chem.*, **118**, 531 (1937).
(8) Sadtler Standard NMR Spectra No. 6065.
(9) R. L. Hamill, *J. Antibiot.*, **29**, 76 (1976).
(10) E. Gross and H. H. Kiltz, *Biochem. Biophys. Res. Commun.*, **50**, 559 (1973).
(11) P. J. Davis, M. Gustafson, and J. P. Rosazza, *Biochim. Biophys. Acta*, **385**, 133 (1975).
(12) H. D. Tresner, J. H. Korshalla, A. A. Fantini, J. D. Korshalla, J. D. Kirby, J. J. Goodman, R. A. Kele, A. J. Shay, and D. B. Borders, *J. Antibiot.*, **31**, 394 (1978).
(13) L. D. Boeck, M. M. Hoehn, T. H. Sands, and R. W. Wetzel, *J. Antibiot.*, **31**, 19 (1978).
(14) J. C. Sheehan, D. Mania, S. Nakamura, J. A. Stock, and K. Maeda, *J. Am. Chem. Soc.*, **90**, 462 (1968).
(15) R. Q. Thompson and M. S. Hughes, *J. Antibiot., Ser. A.*, **16**, 187 (1963).
(16) N. Tsuji and M. Kobayashi, *J. Antibiot.*, **31**, 939 (1978).
(17) G. R. Delbierre, F. W. Eastwood, G. E. Grean, D. G. I. Kingston, P. S. Sarin, Lord Todd, and D. H. Williams, *J. Chem. Soc. C*, **1966**, 1653.

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Prodrugs as Drug Delivery Systems XXV: Hydrolysis of Oxazolidines—A Potential New Prodrug Type

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Abstract □ The hydrolysis kinetics of several oxazolidines derived from (−)-ephedrine and various aldehydes and ketones were studied to assess their suitability as prodrug forms for β-amino alcohols and/or carbonyl-containing compounds. The oxazolidines were found to undergo a facile and complete hydrolysis in the pH range of 1–11 at 37°. The hydrolysis rates were subject to general acid–base catalysis by buffer substances and depended strongly on pH. Most oxazolidines showed sigmoidal pH–rate profiles with maximum rates at pH > 7–7.5. At pH 7.40 and 37° the following half-lives of hydrolysis for the various ephedrine oxazolidines were found: 5 sec (formaldehyde), 18 sec (propionaldehyde), 5 min (benzaldehyde), 5 sec (salicylaldehyde), 30 min (pivalaldehyde), 4 min (acetone), and 6 min (cyclohexanone). The reaction rates in neutral and basic solutions were shown to decrease with increasing steric effects of the substituents derived from the carbonyl component and to decrease with increasing basicity of the oxazolidines. The oxazolidines are weaker bases (pK_a 5.2–6.9) than the parent β-amino alcohol and more lipophilic at physiological pH. It is suggested that oxazolidines can be considered as potentially useful prodrug candidates for drugs containing a β-amino alcohol moiety or carbonyl groups.

Keyphrases □ Ephedrine—oxazolidine derivatives, potential prodrugs for β-amino alcohols and carbonyl-containing compounds □ Oxazolidines—potential prodrugs for β-amino alcohols and carbonyl-containing compounds, ephedrine □ Prodrugs—potential, oxazolidine derivatives, for β-amino alcohols and carbonyl-containing compounds, ephedrine

Bioreversible derivatization of drug substances to produce prodrugs with altered physicochemical properties can improve substantially both drug efficacy and safety (1–3). As a part of current studies involving new chemical approaches (4, 5), an investigation was carried out to obtain prodrug candidates for the β-amino alcohol moiety and/or carbonyl groups (aldehydes and ketones). There are several drugs containing a β-amino alcohol moiety (*e.g.*, various sympathomimetic amines and β-blockers) which may exhibit delivery problems, *e.g.*, due to unfavorable solubility or lipophilicity characteristics. For this moiety no prodrug types have apparently been described, and likewise, only few bioreversible derivatives of carbonyl-containing drugs have been explored (6, 7). We recently suggested (8) that oxazolidines should be considered as potentially useful prodrug candidates for β-amino alcohols or drugs containing carbonyl groups. Oxazolidines (II and III) derived from (−)-ephedrine (I) and benzaldehyde or salicylaldehyde were found to undergo a facile and quantitative hydrolysis in the pH range of 1–11, the half-lives of hydrolysis at pH 7.4 and 37° being 5 min (II) and 5 sec (III). To further explore the potential of oxazolidines as prodrug types and to delineate some structure–activity relationships, this study has been extended to include oxazolidines derived from (−)-ephedrine and the ketones acetone and cyclohexanone, as well as the aliphatic aldehydes formaldehyde, propionaldehyde, and pivalaldehyde. In the present paper the kinetics of hydrolysis of these oxazolidines (IV–VIII) are described along with data for the lipophilicity of the compounds.

EXPERIMENTAL

Chemicals—The oxazolidines IV–VIII were prepared by treating (−)-ephedrine[1] with the appropriate aldehyde or ketone, according to previously described procedures (9–11), and these materials were purified by distillation *in vacuo*. The boiling or melting points observed agreed with those previously reported (9–11), and satisfactory elemental analysis data (C, H, and N) were obtained. Buffer substances and all chemicals or solvents were of reagent grade.

Kinetic Studies—All rate studies were performed in aqueous buffer solutions at 37.0 ± 0.2°. The buffers used were hydrochloric acid, formate, acetate, phosphate, borate, and carbonate solutions. A constant ionic strength (μ) of 0.5 was maintained for each buffer by adding a calculated amount of potassium chloride. The rates of hydrolysis were followed by one or more of three methods depending on the reaction rate.

Direct UV spectrophotometry—In this method the progress of decomposition of the oxazolidines was followed spectrophotometrically[2] by recording the decrease in absorbance at 220 nm. At this wavelength the absorption of substrate and products differed maximally. Reactions were performed in 2.5-ml aliquot portions of buffer solutions in a thermostated quartz cell and were initiated by adding 20 μl of a stock solution of the oxazolidines in acetonitrile to give a final concentration of ~5×10^{-4} *M*. Rate constants were calculated from the slopes of linear plots of log ($A_t - A_\infty$) against time, where A_t and A_∞ are the absorbance

[1] AG Fluka, Switzerland.
[2] Zeiss PMQ II equipped with a thermostated cell compartment.

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