Structure of Obafluorin: An Antibacterial β-Lactone from *Pseudomonas fluorescens*

Adrienne A. Tymiak,* Catherine A. Culver, Mary F. Malley, and Jack Z. Gougoutas

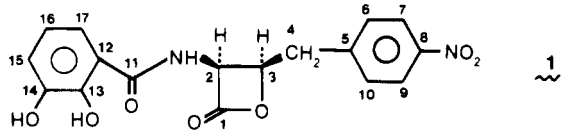
The Squibb Institute for Medical Research, Princeton, New Jersey 08540

Received October 7, 1985

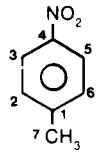
Fermentation of *Pseudomonas fluorescens* (ATCC 39502) produces obafluorin (1), a novel β-lactone antibiotic. Isolation and structure determination of obafluorin are described. The skeleton and relative stereochemistry of 1 were established by using nuclear magnetic resonance and mass spectrometric and X-ray crystallographic analyses. The absolute configuration was deduced from enzymatic degradation of an amino acid fragment produced by hydrolysis of 1. Structural and biological aspects of obafluorin are discussed.

The search for novel β-lactam antibiotics from nature has been pursued with vigor since the discovery of penicillin. Originally, these antibiotics were thought to be strictly fungal metabolites. Later, β-lactam antibiotics were isolated from actinomycetes.[1] Most recently, with the development of a sensitive β-lactamase induction assay, Sykes and co-workers[2,3] were able to demonstrate the production of various β-lactam antibiotics in bacterial fermentations. Their discovery ultimately led to the first clinically useful monocyclic β-lactam, aztreonam.[4]

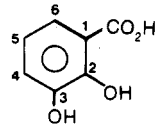
During the course of screening bacteria with this induction assay, it became evident that β-lactones with little or no inherent antibiotic activity could also result in "false" positives.[5] Upon isolation, one lead from these screening efforts proved to be an elaborate β-lactone (1) with unprecedented biological activity.[6] Here we report the structure of obafluorin (1).



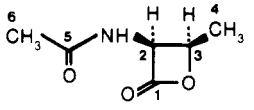
1



2



3



4

Isolation and Structure Elucidation

Pseudomonas fluorescens SC12936 (ATCC 39502) was isolated from a plant specimen collected near Obal Nurseries, Princeton, NJ. Details of the producing organism, its fermentation, and subsequent biological testing of pure 1 are presented elsewhere.[6]

For the purpose of isolation, obafluorin was tracked by its antibiotic activity against *Bacillus licheniformis* SC9262. However, conventional paper disk agar diffusion assays and thin-layer bioautographic techniques proved unsuitable. Binding to paper, streaking during TLC, decomposition in methanolic or basic solution (with a half-life of minutes), and insolubility in aqueous acids were characteristics of obafluorin. As a result, antibiotic zone sizes for 1 were limited by poor diffusion and hydrolysis and did not reflect the amount assayed. Semiquantitative measurement of obafluorin purity was only achieved by determining the minimum dose of 1 required to produce visible antibiosis on *B. licheniformis* seeded agar plates.

After the fermentation was terminated by centrifugation, the clear supernatant was acidified and extracted with ethyl acetate. The organic extract was dried in vacuo and the residue triturated with acetonitrile. Isocratic reversed-phase chromatography of the soluble portion on MCI GEL CHP20P resin served to purify obafluorin. Column fractions containing 1 were combined and dried, providing an amorphous, cream-colored solid. At this stage, obafluorin eluted as one sharp peak from a reversed-phase (C_{18}) HPLC column.

Preliminary characterization was performed on amorphous obafluorin. From the IR spectrum of 1, a β-lactone moiety (1825 cm^{-1}) and nitro group (1520, 1350 cm^{-1}) were readily inferred. The critical role played by the β-lactone ring in antibiosis was evidenced by a complete loss of *Bacillus* inhibition upon hydrolysis of 1 with base. Low-voltage paper electrophoresis of 1 and bioautography suggested a weakly acidic functionality. This acid was identified as a phenol by its characteristic bathochromic shift in the UV spectrum of 1 in basic (275 nm) as compared to neutral (258 nm) acetonitrile solution.

The skeleton of obafluorin was deduced mainly from NMR and mass spectral data. Two aromatic spin systems and one more complicated system containing the β-lactone were identified by a comparison of NMR data for 1 and model compounds 2–4 (Table I). Assignment of the β-lactone ring juncture as cis followed from the measured J_{H2-H3} of 6.5 Hz (CD_3CN).[8] A molecular weight of 358 daltons was derived from FAB mass spectrometry (M + H, m/z 359; M − H, m/z 357). High-resolution measurements indicated a molecular formula of $C_{17}H_{14}N_2O_7$. Fragmentation of 1 and of its base-hydrolysis product 5, as illustrated in Scheme I, provided the linear connectivity for the subunits derived from NMR data. Direct evidence was also obtained by characterizing 3 and 6 from an acid hydrolysate of 1 (Scheme II). NMR data for obafluorin derivatives including the methanolysis product 7 and triacetyl derivative 8 are summarized in Table II.

The remaining question of absolute configuration at the β-lactone methine carbons [C2(*S*), C3(*R*) or C2(*R*), C3(*S*)] was addressed by enzymatic methods. Control experiments were conducted first using L and D amino acid oxidase with L- and D,L-allothreonine.[9] "Chiral 6" (with the

(1) Nagarajan, R.; Boeck, L. D.; Gorman, M.; Hamill, R. L.; Higgins, C. E.; Hoehn, M. M.; Stark, W. M.; Whitney, J. G. *J. Am. Chem. Soc.* **1971**, *93*, 2308–2310.
(2) Sykes, R. B.; Wells, J. S. *J. Antibiot.* **1985**, *38*, 119–121.
(3) Sykes, R. B.; Parker, W. L.; Wells, J. S. In "Trends in Antibiotic Research. Genetics, Biosynthesis, Actions and New Substances"; Umezawa, H., Ed.; Japan Antibiotics Research Association: Tokyo, 1982; pp 115–124.
(4) Cimarusti, C. M.; Sykes, R. B. *Chem. Br.* **1983**, 302–303.
(5) Wells, J. S.; Hunter, J. C.; Astle, G. L.; Sherwood, J. C.; Ricca C. M.; Trejo, W. H.; Bonner, D. P.; Sykes, R. B. *J. Antibiot.* **1982**, *35*, 814–821.
(6) Wells, J. S.; Trejo, W. H.; Principe, P. A.; Sykes, R. B. *J. Antibiot.* **1984**, *37*, 802–803.
(7) Parker, W. L.; Rathnum, M. L.; Liu, W.-C. *J. Antibiot.* **1982**, *35*, 900–902.
(8) Mulzer, J.; Kerkmann, T. *J. Am. Chem. Soc.* **1980**, *102*, 3620–3622.

0022-3263/85/1950-5491$01.50/0

Table I. ^1H and ^{13}C NMR Chemical Shifts for Reference Compounds 2–4 in Comparison to Obafluorin (1)

ref compds									obafluorin[a] (1)		
2[b]			3[c]			4[d]					
posn	^1H	^{13}C	posn	^1H	^{13}C	posn	^1H	^{13}C	posn	^1H	^{13}C
1		146.1							5		146.18
2, 6	7.30	129.9							6,10	7.51	129.47
3, 5	8.10	123.5							7,9	8.09	122.70
4		146.4							8	*e*	148.57
7		21.4							4		34.88
			1		113.30				12		113.25
			2		151.28				13		145.49
			3		146.73				14		143.77
			4	7.3	119.67				15	7.17	116.67
			5	6.65	121.48				16	6.82	118.10
			6	7.05	121.40				17	7.04	118.70
						1		169.6	1		169.25
						2	5.62	58.9	2	5.74	58.45
						3	4.90	75.0	3	5.05	76.81
						5		170.3	11		166.80
						NH	6.80		NH	8.12	

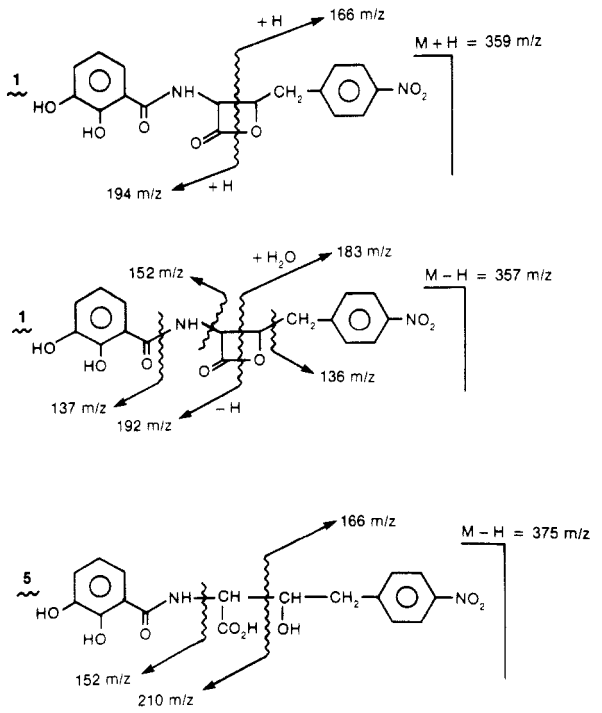
[a] ^1H data for 1 obtained in CD_3CN and ^{13}C data obtained in CD_3COCD_3. Chemical shift assignments are empirical. [b] Data obtained from the Sadtler Standard Spectra compilation: ^1H spectrum #677, $CDCl_3$; ^{13}C spectrum #632, $CDCl_3$. [c] ^1H data from "The Aldrich Library of NMR Spectra", spectrum #12,620-9 in Me_2SO-d_6 + $CDCl_3$; ^{13}C data collected in CD_3COCD_3 and assigned on the basis of predicted chemical shifts. [d] Data collected in $CDCl_3$ (see ref 7). [e] See Table II.

Table II. ^1H NMR Data[a] for Obafluorin (1) and Its Derivatives 5, 7, and 8

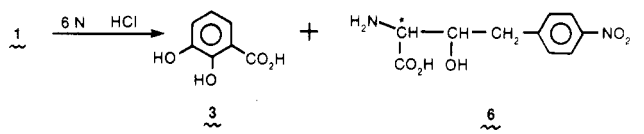
	1 (CD_3CN)		5 (CD_3COCD_3)		7 (CD_3COCD_3)[c]		8 (CD_2Cl_2)	
posn[b]	δ	J	δ	J	δ	J	δ	J
H2	5.74	6.8, 8.0	4.84	m	4.83	m	5.67	br d
H3	5.05	m	4.62	m	4.58	m	4.84	m
H4a	3.20	5.1, 15	3.14	5.1, 13.6	3.14	dd	3.10	13,15
H4b	3.37	9.1, 15	3.05	8.6, 13.5	3.05	dd	3.40	9.1, 15
H6/H10	7.51	9.1	7.61	8.8	7.60	8.8	7.45	8.4
H7/H9	8.09	8.8	8.16	8.4	8.15	8.8	8.18	8.8
N-H	8.12	8.0	7.88	7.0	8.09	7.3		
H15	7.17	8.1	7.41	8.1	7.46	8.0	7.35	7.0
H16	6.82	8.1, 8.1	6.80	7.6, 7.7	6.80	8.0, 8.1	7.42	dd
H17	7.04	8.1	7.02	7.7	7.02	7.7	7.35	7.0
OCH_3					3.70	s		
CH_3CO							2.29, 2.32[d]	s
CO_2H			12.21	br s				

[a] Chemical shifts (δ) relative to protonated solvent peaks and coupling constants (J) reported in Hertz. [b] Positions based on numbering system assigned to 1. [c] Broad spectrum obtained, but approximate J values are comparable to those tabulated for 1. [d] Nine protons observed, presumably due to six O-Ac (δ 2.32) and three N-Ac (δ 2.29) protons.

Scheme I



Scheme II



natural chirality at the α-carbon intact but the configuration at the β-carbon presumably scrambled[10]) was generated by 6 N HCl hydrolysis of obafluorin. A portion of the purified amino acid **6** was racemized at the α-carbon by treatment with salicylaldehyde[11] to afford "epimerized **6**". Both obafluorin-derived amino acid samples were then enzymatically deaminated. Thus, having previously assigned the relative configuration, the [C2(*S*), C3(*R*)] absolute configuration for **1**, analagous to L-threonine, could be assigned on the basis of enzymatic digestion results (Table III). This is consistent with previously reported pyrocatechuamides which all have the L configuration.

(9) Relative to their β-hydroxy epimers, D- and L-threonine are known to be sluggish substrates for the respective amino acid oxidase. For a discussion, see: Greenstein, J. P.; Winitz, M. "Chemistry of the Amino Acids"; Wiley: New York, 1961; pp 187–190.

(10) Hine, J. "Physical Organic Chemistry"; McGraw-Hill: New York, 1962; pp 280–282.

(11) Yamada, S.; Hongo, C.; Yoshioka, R.; Chibata, I. *J. Org. Chem.* **1983**, *48*, 843–846.

Table III. Amino Acid Oxidase Deamination Experiment

substrate	enzyme prep L	D	L + D
L-allothreonine	+[b]	−[c]	ND[d]
D,L-allothreonine	+−[e]	+−	+
"chiral 6"	+	−	ND
"epimerized 6"	+−	ND	+

[a] Enzyme treatment followed by low-voltage electrophoresis of the reaction mixture (pH 9.2) and ninhydrin spray visualization of the residual amino acid. [b] + = complete loss of ninhydrin positive material. [c] − = no apparent deamination. [d] ND = not determined. [e] +− = visible decrease in ninhydrin stain intensity.

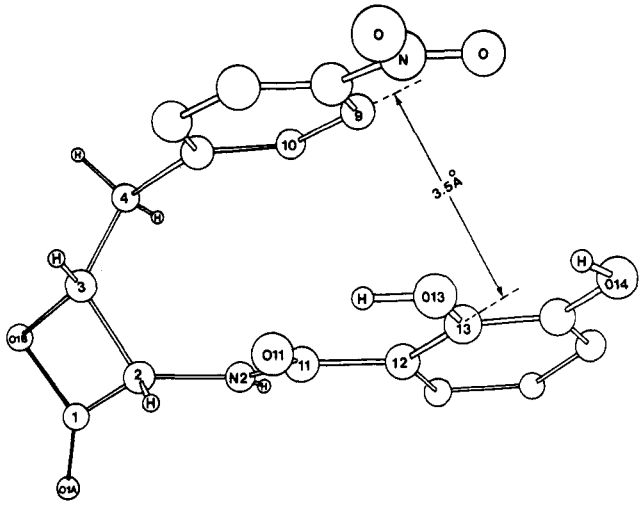


Figure 1. Perspective drawing of the solid-state conformation of 1. The aromatic hydrogens and the acetonitrile in the crystal structure are not shown.

Previously,[7] another bacterially produced β-lactone (4) was shown to have the [C2(*S*), C3(*R*)] configuration via hydrolysis to L-threonine.

When allowed to stand in wet acetonitrile, obafluorin crystallized as a 1:1 solvate with acetonitrile (mp 109–113 °C). A single-crystal X-ray study confirmed the proposed skeleton and relative stereochemistry of 1 and provided a precise measure of the geometry of the β-lactone moiety (Figure 1). Slight buckling of the lactone ring (ring torsional angles are alternately ±5°) and some angular distortions at C2 and C3 result in torsional angle N2–C2–C3–C4 = 9.8° for the cis substituents. Virtually identical ring conformations have been found in the crystal structures of several similarly substituted monobactams that have been determined in our laboratories, and it is clear that β-lactones and β-lactams may be conformationally isosteric. The ring bond lengths and angles in 1 (starting at C1 and proceeding clockwise in Figure 1) are 1.366 (4), 1.482 (3), 1.531 (4), and 1.511 (4) Å and 93.8 (2), 92.3 (2), 88.6 (2), and 85.0 (2)°.

Recent surveys of the geometries of lactones and lactams indicate consistent trends in bond angle differences between β-lactones and β-lactams.[12-14] Whereas in 1 and other β-lactones the endocyclic angle C3–O1B–C1 is somewhat smaller than the endocyclic angle O1B–C1–C2, this apparent trend is clearly reversed for the corresponding angles (94.4 and 91.7°, respectively) in published β-lactam structures. A somewhat larger spread is apparent

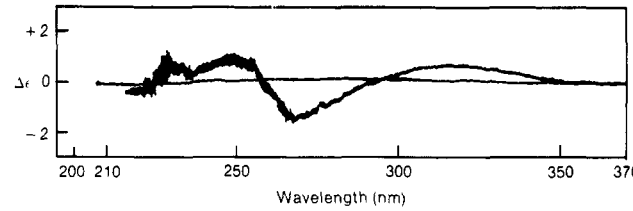


Figure 2. Circular dichroic (CD) spectrum of 1. The CH_3CN solution (1.2×10^{-4} M) was measured at 52×10^{-1} sensitivity, 1-s time constant, 10 nm/cm scan speed, and 1 cm/min chart speed.

in our unpublished[15] crystallographic structural database of *nonfused* β-lactams having exocyclic C, O, P, and S substituents on the lactam nitrogen atom (96.4, 90.7°; averaged over 24 structures with $R < 0.09$). However, the greatest angular differences between β-lactones and β-lactams involve the exocyclic bond angles of the carbonyl group. The relatively large difference (C2–C1–O1A) – (O1B–C1–O1A) = 139.2 – 127.0 = 12.2° in 1 and other β-lactones is less pronounced for the corresponding angles in β-lactams, Δ = +4.0° (Δ = +3.4° in our structural database of nonfused β-lactams).

In the solid-state conformation of obafluorin, the nitrophenyl group and O1B are anti with respect to the C3–C4 bond, as are C11 and C1 with respect to the N2–C2 bond. The β-lactone substituents thus are extended in nearly parallel (2.5°) stacked planes, with closest contacts between aromatic carbon atoms occurring between C13 and C9 (3.458 Å) and C12 and C10 (3.523 Å). The catechol ring is rotated by 15.8° from the plane of the *Z* amide link such that the intramolecular O13···O11 and O13···O14 distances are 2.563 and 2.719 Å, respectively. In addition to these two possible intramolecular hydrogen-bonding interactions, an intermolecular hydrogen bond may occur between O14 and O11 (2.750 Å). The amide proton is hydrogen bonded to the nitrogen atom of the acetonitrile (N···N = 2.966 (4) Å).

Although the solution conformation of obafluorin has not been determined, it seems likely that some intramolecularly stacked arrangements of the two aromatic β-lactone substituent persist in solvents. These may give rise to the observed CD spectrum of 1 (Figure 2) through exciton coupling[16] of the aromatic chromophores.

Discussion and Biological Activity

The β-lactone moiety is scarce among natural products but is usually associated with biological activity. To date, however, only a few X-ray structure analyses of the β-lactone moiety have been described.[17-19] Similarly, nitro-containing natural products are uncommon and are often biologically active.[20] In contrast, 2,3-dihydroxybenzamides form the basis of a class of microbial siderophores.[21-25]

(12) Schweizer, W. B.; Dunitz, J. D. *Helv. Chim. Acta* **1982**, *65*, 1547–1554.
(13) Chakrabarti, P.; Dunitz, J. D. *Helv. Chim. Acta* **1982**, *65*, 1555–1562.
(14) Norskov-Lauritsen, L.; Burgi, H.-B.; Hoffmann, P.; Schmidt, H. R. *Helv. Chim. Acta* **1985**, *68*, 76–82.
(15) Malley, M. F.; Gougoutas, J. Z., unpublished work.
(16) Harada, N.; Nakanishi, K. "Circular Dichroic Spectroscopy, Exciton Coupling in Organic Stereochemistry"; University Science Books: Mill Valley, CA, 1983.
(17) Kikuchi, H.; Tensho, A.; Shimizu, I.; Shiokawa, H.; Kuno, A.; Yamada, S.; Fujiwara, T.; Tomita, K. *Chem. Lett.* **1983**, 603–606.
(18) Sakabe, N.; Hirata, Y.; Furusaki, A.; Tomiie, Y.; Nitta, I. *Tetrahedron Lett.* **1965**, 4795–4796.
(19) Ogihara, Y.; Asada, M.; Iitaka, Y. *J. Chem. Soc., Chem. Commun.* **1978**, 364–365.
(20) Eckstein, Z. *Wiad. Chem.* **1981**, 579–618 (Pol.).
(21) Griffiths, G. L.; Sigel, S. P.; Payne, S. M.; Neilands, J. B. *J. Biol. Chem.* **1984**, *259*, 383–385.
(22) Peterson, T.; Falk, K.-E.; Leong, S. A.; Klein, M. P.; Neilands, J. B. *J. Am. Chem. Soc.* **1980**, *102*, 7715–7718.
(23) Corey, E. J.; Bhattacharyya, S. *Tetrahedron Lett.* **1977**, 3919–3922.

Obafluorin, which combines all the above structural characteristics, is a weak broad-spectrum antibiotic when assayed by disk diffusion.[6] In a standard agar dilution assay, where hydrolysis of the β-lactone is inevitable, **1** lost virtually all of its antibiotic activity. In spite of its lability, however, obafluorin did provide some protection to mice infected with a recent clinical isolate, *Streptococcus pyogenes* SC12960 (ED_{50} = 50 mg/kg by systemic administration). This unexpected result in vivo prompted closer inspection of the effects of **1** in biological systems.

Microscopic examination revealed cell elongation in *Escherichia coli* grown at sublethal doses of **1**. Additionally, studies with β-lactamases from three bacterial sources demonstrated that **1** was indeed susceptible to enzymatic hydrolysis, becoming the first example of a β-lactone substrate for a β-lactamase.[6] These observations suggest that obafluorin is acting in some specific manner reminiscent of β-lactam antibiotics, rather than as a general acylating agent. Close structural similarities between **1** and several monocyclic β-lactam antibiotics (monobactams), as evidenced by X-ray crystallographic studies, support this conjecture. In conclusion, the specific mode of action of obafluorin is still unknown although the biological activity appears to be a consequence of the β-lactone ring.

Experimental Section

Melting points were not corrected. NMR spectra were obtained with a Jeol GX400 spectrometer; all chemical shifts (δ) are reported downfield from an internal tetramethylsilane (0 ppm, organic solvents) or *tert*-butyl alcohol (1.25 ppm, D_2O) standard. A modified Extranuclear Simulscan instrument was used to obtain CI mass spectra while FAB mass spectra were measured on a VG-ZAB IF mass spectrometer. Low-voltage paper electrophoresis was performed at 250 V, 12 V/cm for 1 h; mobility was scored relative to vitamin B_{12} (0) and *p*-nitrobenzenesulfonate anion (+1.00). Bioassays were routinely carried out by sorbing fractions onto 6.3-mm Whatman #4 paper disks and applying these to *B. licheniformis* SC9262 seeded agar plates. Antibiotic zones were measurable after 4 h of incubation at 37 °C.

Isolation of Obafluorin (1). The fermentation broth (5 L) was centrifuged at 62500*g* to sediment bacteria. The supernatant was acidified to pH 3 and extracted with ethyl acetate (3 × 1.75 L). The combined organic layers were concentrated to dryness and then triturated with 20 mL of CH_3CN to give a soluble portion containing **1** that dried in vacuo to an oily residue (459.3 mg). The residue was dissolved in 4.6 mL of CH_3CN–H_2O–TFA (55:45:0.1) and chromatographed a half-portion at a time on a 90-mL column of MCI GEL CHP20P resin (Mitsubishi Chemical Industries, Ltd.) packed in the same solvent. The active fractions from both columns were combined and dried in vacuo, providing 139.4 mg of amorphous obafluorin (**1**): UV (EtOH) λ_{max} 215 nm (ε 25 000), 258 (18 500), 325 (5000); UV (EtOH–0.1 N HCl) λ_{max} 258 nm (ε 15 500), 325 (3500); UV (EtOH–0.1 N NaOH) λ_{max} 275 nm (ε 17 300), 330 (6200); $[\alpha]_D$ +116° (*c* 0.1, CH_3CN); IR (KBr) 3800–2900, 1825, 1700, 1600, 1520, 1350 cm^{-1}; ^1H NMR (CD_3CN) δ 11.7 (br s), 6.8 (br s), see Table II; ^{13}C NMR (CD_3CN) δ 170.5, 168.2, 149.5, 147.3, 146.3, 144.7, 130.5 (2 C), 124.0 (2 C), 119.8, 119.5, 114.4, 78.0, 59.6, 36.0; ^{13}C NMR (CD_3COCD_3) see Table I; FABMS (– ion, tetramethylenesulfone) *m/z* 717 (2M – H), 733 (2M + H_2O – H), 357 (M – H), 313 (M – CO_2 – H); HRFABMS (– ion) *m/z* 357.080 ($C_{17}H_{13}N_2O_7$ requires *m/z* 357.072); FABMS (+ ion) *m/z* 359 (weak); CIMS (H_2O) *m/z* 359, 341, 194, 184, 166.

X-ray Diffraction of 1. Crystalline **1**·CH_3CN solvate was obtained by slow evaporation of an acetonitrile–water–trifluoroacetic acid solution. The unit cell parameters were obtained through a least-squares analysis of the experimental diffractometer settings of 15 reflections with $2\theta > 45°$ using Cu Kα monochromatic radiation (λ = 1.5418 Å): *a* = 7.519 (2), *b* = 11.539 (2), *c* = 10.805 (2) Å. Space group $P2_1$ was assigned on the basis of systematic absences 0*k*0 (*k* = 2*n* + 1). The crystal density, D_{obsd} = 1.40 cm^3 (D_{calcd} = 1.415 for *Z* = 2, $C_{19}H_{17}N_3O_7$) was measured by flotation in hexane–carbon tetrachloride mixtures. A total of 1356 intensities were measured on a Syntex $P2_1$ diffractometer at 23 °C with the θ–2θ variable scan technique and were corrected only for Lorentz–polarization factors. The structure was solved by direct methods and refined by full-matrix least-squares analysis on the basis of 1198 "observed" reflections for which $I \geq 3\sigma(I)$.

The hydrogen positions were evident in difference maps. Only the hydrogens on O13, O14, and N2 were introduced in observed positions; all other hydrogens were introduced in idealized positions. The scattering from all of the hydrogens was taken into account in the terminal stages of refinement. The least-squares weights, $w = \sigma^{-2}(F_o)$ were calculated with the assumption that $\sigma^{-2}(I) = \epsilon^2 + (\rho I)^2$ where ε is a statistical counting error and ρ = 0.02. The refinements (assuming the absolute configuration shown in Figure 1, holding the *y* coordinate of C1 fixed and assuming anisotropic motion for all N, C, and O atoms) converged at *R* = 0.034, R_w = 0.038. No statistically significant difference was evident in refinements based on the enantiomeric structure. The final difference map contained no significant features. Tables of the atomic coordinates, thermal parameters, bond distances, and bond angles are included as supplementary material.

Mild Base Hydrolysis of 1. Obafluorin (2 mg) was dissolved in 0.05 N NaOH (150 μL). After standing at room temperature for 15 min, the solution was acidified with 0.05 N HCl and then extracted with ethyl acetate (3 × 200 μL). Concentration of the combined organic extracts provided 1.8 mg of amorphous **5**: UV (EtOH) λ_{max} 215 nm (ε 20 000), 258 (15 800); UV (EtOH–0.1 N HCl) λ_{max} 256 nm (ε 15 400); UV (EtOH–0.1 N NaOH) λ_{max} 270 nm (ε 17 700), 330 (8600); $[\alpha]_D$ +40° (*c* 0.14, CH_3CN); IR (KBr) 3800–2900, 1750, 1650, 1530, 1350 cm^{-1}; ^1H NMR (CD_3COCD_3) δ 12.25 (s), see Table II; FABMS (– ion, glycerol) *m/z* 375, 210, 166, 152; HRFABMS (– ion) *m/z* 375.079 ($C_{17}H_{15}N_2O_8$ requires *m/z* 375.082); FABMS (+ ion) *m/z* 377, 216, 199, 167, 165, 163, 137.

Methanolysis of 1. Obafluorin (2.9 mg) was treated with triethylamine (12.5 μL) in methanol (1.25 mL) at room temperature for 30 min. The solution was evaporated under a stream of dry nitrogen, yielding 2.9 mg of **7**: UV (EtOH) λ_{max} 215 nm (ε 20 300), 258 (9000); IR (CH_3CN) 3620, 3540, 3040–2920, 1750, 1710, 1640, 1600, 1520, 1350 cm^{-1}; ^1H NMR (CD_3COCD_3) see Table II; FABMS (– ion, thioglycerol) *m/z* 391; FABMS (+ ion) *m/z* 389.

Acetylation of 1. Acetic anhydride and pyridine (2:1, 1.25 mL) were added to 2.5 mg of **1** with stirring. After 1 h at room temperature the mixture was evaporated under a stream of nitrogen. Preparative TLC (Et_2O) of the residual oil gave 1.6 mg of amorphous **8**: UV (EtOH) λ_{max} 215 nm (ε 21 800), 253 (10 200), 280 (9200); IR (CH_3CN) 1840, 1780, 1710, 1680, 1600, 1530, 1350 cm^{-1}; ^1H NMR (CD_2Cl_2) see Table II; CIMS (H_2O) *m/z* 485, 443, 425, 401, 265, 247, 221, 197, 179.

Degradation of 1 To Form Amino Acid 6. Oblafluorin (2 mg) was degraded with constant-boiling HCl (6 N, 200 μL) at 110 °C for 17 h. The reaction mixture was evaporated with nitrogen gas and then purified by chromatography on AG MP 50 (H^+, 200–400 mesh). Pyrocatechuic acid (**3**) was eluted with CH_3CN–H_2O–TFA (50:50:0.1), and then 1 M NH_4OH was used to elute the mixture of C-3 epimers of **6**: LVE –0.28 (pH 2.3), 0.0 (pH 4.5), +0.08 (pH 7.0), +0.75 (pH 9.2); ^1H NMR (D_2O) δ 8.22 (d, *J* = 8.5 Hz), 7.53 (d, *J* = 8.5 Hz), 4.14 (m), 3.53 (d, *J* = 4.1 Hz), 3.31 (d, *J* = 4.4 Hz), 3.06 (dd, *J* = 4.0, 13.8 Hz), 2.90 (dd, *J* = 10.0, 14.1 Hz); FABMS (+ ion, thioglycerol) *m/z* 241; FABMS (– ion) *m/z* 239.

General Procedure for Amino Acid Oxidase Transaminations. A modified protocol was developed on the basis of previous work with D and L amino acid oxidases.[26,27] Each amino acid was dissolved in distilled water (1 mg/mL), and portions (50 μL) were diluted with 20 μL 0.1 M EDTA in sodium 1 M phosphate buffer (pH 8.3). To each buffered amino acid were added 1.5 units of the appropriate amino acid oxidase (Sigma) in 20 μL of buffer and 10 μL of a 2 mg/mL catalase (Sigma) solution. The

(24) O'Brien, I. G.; Gibson, F. *Biochim. Biophys. Acta* **1970**, *215*, 393–402.
(25) Corbin, J. L.; Bulen, W. A. *Biochemistry* **1969**, *8*, 757–762.
(26) Holme, D. J.; Goldberg, D. M. *Biochim. Biophys. Acta* **1975**, *377*, 61–70.
(27) Hardy, M. J. *Anal. Biochem.* **1974**, *57*, 529–533.

mixtures were incubated at 37 °C for 16 h and were periodically analyzed by LVE followed by ninhydrin visualization of the unreacted amino acid. See Table III for specific results.

Acknowledgment. We are grateful to the Squibb Institute Analytical Department and especially to Dr. M. A. Porubcan and Dr. S. E. Unger for their valuable assistance during the course of this work. Thanks are also due to Dr. J. Clark, Dr. K. Tanaka, and Dr. S. Wells for biological testing at Squibb and to Dr. K. Nakanishi for providing the CD measurement at Columbia University. We thank D. D. Lutsky and Dr. W. L. Parker for help in preparation of the manuscript.

Registry No. 1, 92121-68-1; 5, 99268-39-0; 6, 99268-38-9; 7, 99268-40-3; 8, 99268-41-4.

Supplementary Material Available: Table IV, positional and thermal parameters, Table V, bond distances, and Table VI, bond angles (4 pages). Ordering information is given on any current masthead page.

Alkylation of Allylic Derivatives. 10.[1] Relative Rates of Reactions of Allylic Carboxylates with Lithium Dimethylcuprate

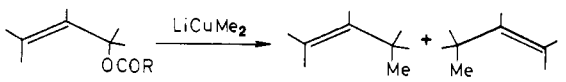
Harlan L. Goering,* Steven S. Kantner,[2] and Earl P. Seitz, Jr.

Samuel M. McElvain Laboratories of Organic Chemistry, Department of Chemistry, University of Wisconsin, Madison, Wisconsin 53706

Received July 19, 1985

Relative rates of reaction of 19 allylic esters with $LiCuMe_2$ in ether have been determined by a competitive reaction technique. The reactivity range for the series is $>3 \times 10^5$. The relationship between structure and reactivity is compatible with a rate-limiting S_N2' oxidative addition to give a σ-allyl copper(III) complex.

In connection with our mechanistic studies of alkylation of allylic carboxylates with organocopper reagents[1] we have examined the relationship between structure and reactivity for alkylation of allylic esters with $LiCuMe_2$ in ether (eq 1). In this study a competitive reaction technique[3] was



(1)

used to determine rate-constant ratios for pairs of allylic carboxylates. This method was chosen instead of attempting absolute rate measurements because of anticipated difficulties with reproducing reaction conditions—reactions are rapid (typically complete in <10 min at 0 °C)[4] and decomposition of the cuprate accompanies the reaction. Absolute rate measurements have been reported for reaction of $LiCuMe_2$ with enones,[5] alkyl tosylates,[6] and iodides.[7] However, the special techniques involved did not appear applicable for the present study.

The competitive method,[3] in which pairs of allylic carboxylates compete for a limited amount of cuprate, is foolproof with regard to comparison of two substrates under identical conditions. The ratio of the rate constant for substrate A (k_A) to that for substrate B (k_B) is given by eq 2 in which A_0 and B_0 are initial amounts and A and

$$\frac{k_A}{k_B} = \frac{\ln (A/A_0)}{\ln (B/B_0)} \qquad (2)$$

B are final amounts.[3] This equation is based on the assumption that the reaction is first order with respect to the allylic substrate. This has been shown to be the case in other work.[8] The order with respect to $LiCuMe_2$ need not be known because any functional dependence on this reagent cancels in the ratio.

The A/A_0 and B/B_0 ratios are fractions of the original amounts of competing esters that remain when the reaction terminates. An important feature of this function is that except for $k_A/k_B = 1$, the $(A/A_0)/(B/B_0)$ ratio tends toward zero ($k_A > k_B$) or infinity ($k_A < k_B$) as A and B approach zero.[3a] Thus accurate rate-constant ratios will only be obtained if an appreciable amount of the more reactive partner remains unreacted. At the same time, the less reactive partner must undergo enough reaction so that the difference between initial and final concentrations is large relative to experimental error. This means that reliable rate-constant ratios are obtained only when comparing substrates of similar reactivity, for example, when the difference in rate constants is <50-fold. In this work a ladder technique was used to span a reactivity range of $>3 \times 10^5$ which corresponds to a difference in free energy of activation ($\Delta\Delta G^{\ddagger}$) of ~7 kcal/mol.

The $LiCuMe_2$ used in these experiments was either prepared directly from cuprous iodide and 2 equiv of methyllithium–lithium bromide complex or by treating well washed methyl copper with 1 equiv of methyllithium–lithium bromide complex. Thus the $LiCuMe_2$ solution contained either 1 or 3 equiv of lithium bromide. The presence of lithium halide salts increases the rate of reaction of $LiCuMe_2$ with alkyl iodides[7] and decreases the rate with enones;[5] however, these salts do not appreciably change the magnitude of rate differences.[5] In this work we observed that $LiCuMe_2$ prepared by the two methods gave similar results.

In the competitive experiments, ethereal $LiCuMe_2$ was added rapidly to an excess of an equimolar mixture of a pair of allylic carboxylates (substrates A and B) and an internal standard in ether at 0 °C. The reaction mixture

(1) Previous paper in this series: Goering, H. L.; Tseng, C. C. *J. Org. Chem.* **1985**, *50*, 1597.
(2) National Science Foundation Fellow, 1977–1980.
(3) (a) Gilliom, R. D. "Introduction to Physical Organic Chemistry"; Addison-Wesley: Reading, MA, 1970; pp 96–99. Melander, L. "Isotope Effects on Reaction Rates"; Ronald Press: New York, 1960; Chapter 3. (b) Walling, C.; Helmreich, W. *J. Am. Chem. Soc.* **1959**, *81*, 1144.
(4) Goering, H. L.; Seitz, E. P., Jr.; Tseng, C. C. *J. Org. Chem.* **1981**, *46*, 5304.
(5) Krause, S. R.; Smith, S. G. *J. Am. Chem. Soc.* **1981**, *103*, 141.
(6) Johnson, C. R.; Dutra, G. A. *J. Am. Chem. Soc.* **1973**, *95*, 7783.
(7) Pearson, R. G.; Gregory, C. D. *J. Am. Chem. Soc.* **1976**, *98*, 4098.
(8) Paisley, S. D., unpublished work in these laboratories.

0022-3263/85/1950-5495$01.50/0