

1.0 mL of AcOH and spin-evaporated in vacuo. The residue was dissolved in 50 mL of H₂O and applied to a column of Amberlite XAD-2 nonionic polymeric absorbant (200 g, 3.5 × 31 cm). The column was eluted with H₂O to give ten 200-mL fractions containing salt-free 12. The first two fractions were contaminated with some NaOAc and were rechromatographed. The combined fractions were spin-evaporated in vacuo, giving 0.350 g (49%) of 12 as a white solid. Three recrystallizations from aqueous EtOH with concentration by boiling with continued addition of C₆H₆ gave 0.155 g (22%) of 12: mp 173–176 °C; TLC (P, solvent 7); UV (0.1 N HCl) λ_{\max} 256 nm (ϵ 14 000), 276 (sh) (9500); UV (0.1 N NaOH) λ_{\max} 257 nm (ϵ 11 900); NMR (Me₂SO-*d*₆) δ 7.77 (s, 1 H, C-8), 6.44 (br s, 2 H, NH₂), 5.31 (s, 2 H, NCH₂O), 4.38 (br s, 1 H, NH), 3.6–3.2 (m, 5 H, 2CCH₂N + OH), 2.7–2.4 (m, 4 H, 2CCH₂O). Anal. (C₁₀H₁₆N₆O₃) C, H, N.

9-[(2-Acetamidoethoxy)methyl]guanine (13). To a stirred solution of 0.64 g (1.9 mmol) of 9 in 30 mL of pyridine and 10 mL of H₂O was added 0.30 g (2.9 mmol) of acetic anhydride. After

15 h at ambient temperature, the reaction was spin-evaporated in vacuo. The residue was dispersed in EtOH and reevaporated to give a solid, which was collected, washed with Et₂O, and dried: yield 0.37 g (74%); mp 246–256 °C. Recrystallization from EtOH gave the analytical sample: yield 0.18 g (36%); mp 240–242 °C dec; TLC (C, solvent 6); UV (0.1 N HCl) λ_{\max} 256 nm (ϵ 12 300); UV (0.1 N NaOH) λ_{\max} 257 nm (ϵ 11 100). Anal. (C₁₀H₁₄N₆O₃) C, H, N.

Acknowledgment. The authors are indebted to Dr. D. J. Bauer and his group for the plaque-inhibition antiviral testing and to Dr. P. Furman for the IC₅₀ against HSV-1. We thank Ms. N. K. Cohn for the Detroit 98 and mouse L cells cytotoxicity tests and Dr. J. A. Fyfe and P. M. Keller for the HSV-1 thymidine kinase results. Dr. B. S. Hurlbert and his staff obtained the elemental analyses and NMR spectra.

Synthesis and β -Lactamase Inhibitory Properties of 2 β -(Chloromethyl)-2 α -methylpenam-3 α -carboxylic Acid 1,1-Dioxide

William J. Gottstein,* Leonard B. Crast, Jr., Robert G. Graham, Ute J. Haynes, and Donald N. McGregor

Research Division, Bristol Laboratories, Division of Bristol-Myers Company, Syracuse, New York 13201.

Received June 15, 1981

Potassium 2 β -(chloromethyl)-2 α -methylpenam-3 α -carboxylate 1,1-dioxide (BL-P2013) and its pivaloyloxymethyl ester were prepared by the conversion of 6-aminopenicillanic acid to *p*-nitrobenzyl 6 α -bromo-2,2-dimethylpenam-3 α -carboxylate 1-oxide, which was rearranged with benzoyl chloride and quinoline to *p*-nitrobenzyl 6 α -bromo-2 β -(chloromethyl)-2 α -methylpenam-3 α -carboxylate in 65% yield. Oxidation and catalytic hydrogenation afforded BL-P2013, which was found to be a potent inhibitor of various bacterial β -lactamases and has been found to protect amoxicillin from β -lactamases in both in vitro and in vivo systems.

The ability of many species of bacteria to produce a β -lactamase which destroys certain β -lactam antibiotics has led to an intensive search for substances that inhibit the action of these protective enzymes. The discovery of clavulanic acid¹ as a potent β -lactamase inhibitor led to the discovery of a variety of new inhibitors which incorporate a β -lactam ring in their structures. Many of these compounds, such as 6-aminopenicillanic acid sulfone,² 6 β -bromopenicillanic acid,³ 6 α -chloropenicillanic acid sulfone,⁴ and various 6 β -(acylamino)penicillanic acid sulfones,⁵ are relatively simple semisynthetic derivatives of 6-aminopenicillanic acid. The most extensively investigated example of this class is penicillanic acid sulfone (CP-45899),⁶ which has been reported to have promising β -lactamase inhibitory properties both in vitro and in vivo. We report here a new example of this class of β -lactamase inhibitor, 2 β -(chloromethyl)-2 α -methylpenam-3 α -carboxylic acid 1,1-dioxide (BL-P2013), which can be prepared from 6-aminopenicillanic acid and which demonstrates high β -lactamase inhibitory activity against a variety of bacterial β -lactamases and which effectively protects amoxicillin from these β -lactamases in both in

Table I. β -Lactamase Inhibitory Properties of Various Compounds against Several Bacterial β -Lactamases

| compd | min protective concn, ^a μg/mL | | |
|--|---|-------------|-------------|
| | <i>K.p.</i> | <i>S.a.</i> | <i>B.f.</i> |
| methicillin sulfone | 125 | >100 | |
| 6 β -chloropenicillanic acid sulfone | 25 | >100 | |
| BL-P2013 | 3.1 | 3.1 | 12.5 |
| CP-45899 | 1.6 | 12.5 | 12.5 |
| clavulanic acid | 0.1 | 0.4 | 6.3 |

^a Ability of a compound to inhibit the hydrolysis of an indicator compound, 7-(phenylacetamido)-3-(2,4-dinitrostyryl)-3-cephem-4-carboxylic acid, by β -lactamases from *Klebsiella pneumoniae* A20634 (TEM) (*K.p.*), *Staphylococcus aureus* A9606 (*S.a.*), and *Bacillus fragilis* A22695 (*B.f.*) by the procedure of C. H. O'Callaghan et al., *Antimicrob. Agents Chemother.*, 1, 283–288 (1972). The MPC is the lowest concentration of compound needed to protect the indicator compound from hydrolysis by the β -lactamases within 30 min under standard test conditions.

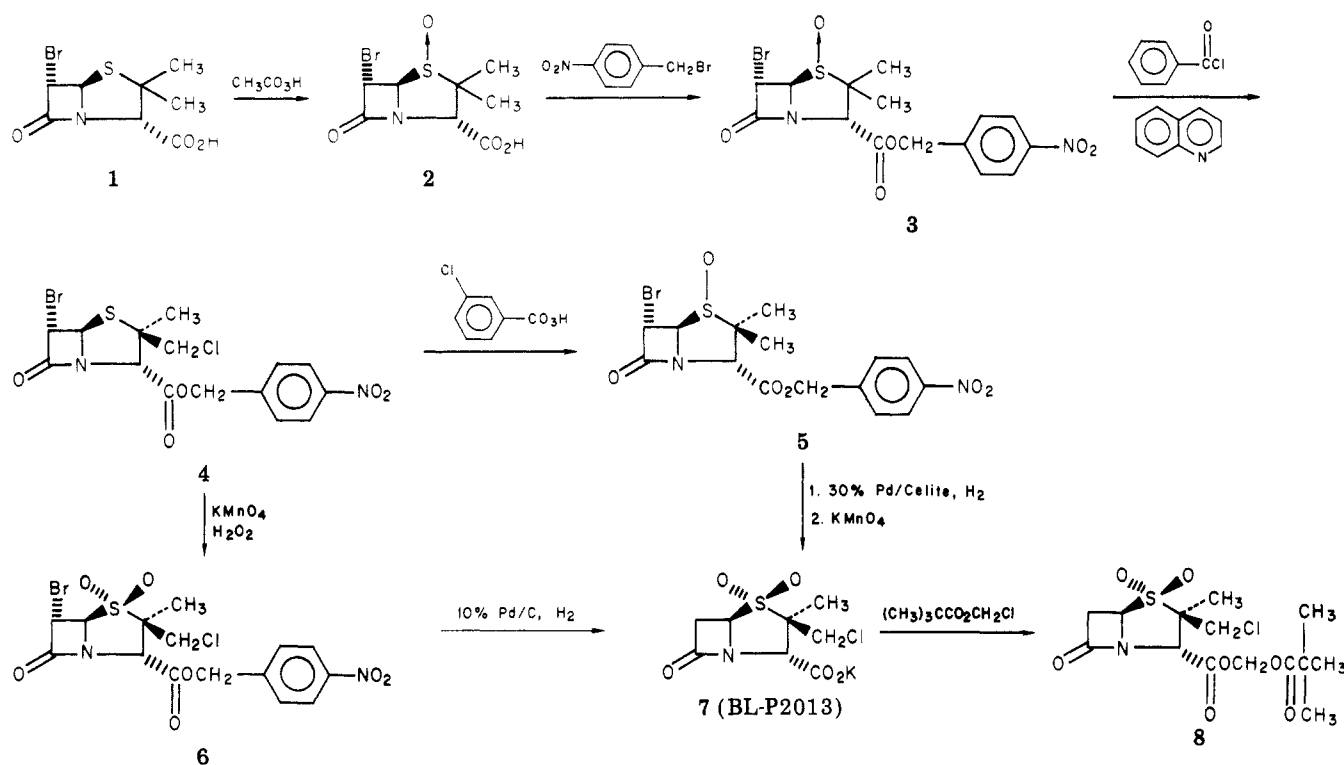
vitro and in vivo test systems.

Chemistry. 6 α -Bromopenicillanic acid (1) was prepared from 6 β -aminopenicillanic acid by the method of Cignarella et al.⁷ by substituting hydrobromic acid for hydrochloric acid and was isolated as a *N,N'*-dibenzylethylenediammonium salt. This was converted to the sulfoxide 2 with peroxycetic acid in methylene chloride and isolated as the potassium salt (Scheme I). Esterification with *p*-nitrobenzyl bromide in dimethylacetamide gave the crystalline *p*-nitrobenzyl ester 3. The rearrangement of

- (1) C. Reading and M. Cole, *Antimicrob. Agents Chemother.*, 11, 852 (1977); C. E. Howarth, A. G. Brown, and T. J. King, *Chem. Commun.*, 266 (1976).
- (2) W. E. Barth, European Patent Application 0008917, 1980; A. R. English, J. A. Retsema, A. E. Girard, J. E. Lynch, and W. E. Barth, *Antimicrob. Agents Chemother.*, 14, 414 (1978).
- (3) V. Knott-Hunziker, B. S. Orlek, P. G. Sammes, and S. G. Waley, *Biochem. J.*, 177, 365 (1979).
- (4) S. J. Cartwright and A. F. Coulson, *Nature (London)*, 278, 361 (1979).
- (5) J. F. Fisher and J. R. Knowles, European Patent Application 0017425, 1980.
- (6) N. Aswapokee and H. C. Neu, *J. Antibiot.*, 31, 1238 (1978).

- (7) G. Cignarella, G. Pifferi, and E. Testa, *J. Org. Chem.*, 27, 2668 (1962).

Scheme I

Table II. Synergistic Combinations of Amoxicillin with Various β -Lactamase Inhibitors

| organism | min inhibitory concn, ^a $\mu\text{g/mL}$ | | | | | |
|-------------------------|---|--|----------|----------|--|-----------------------------------|
| | amoxicillin | amoxicillin + BL-P2013, 1:1 ^b | BL-P2013 | CP-45899 | amoxicillin + CP-45899, 1:1 ^b | clavulanic acid, 2:1 ^b |
| <i>S. aureus</i> A9606 | >125 | 1:1 | 125 | >125 | 2:2 | 32 |
| <i>S. aureus</i> A15091 | >125 | 1:1 | 125 | >125 | 2:2 | 32 |
| <i>S. aureus</i> A20309 | 125 | 2:2 | 125 | >125 | 4:4 | 32 |
| <i>E. coli</i> A9675 | 63 | 2:2 | >125 | 125 | 4:4 | 32 |
| <i>E. coli</i> A21223 | 125 | 2:2 | >125 | >125 | 4:4 | 32 |

^a MIC values were obtained using an agar dilution method whereby organisms were deposited onto medicated agar plates by the replication device of Steers et al., *Antibiot. Chemother.*, 9, 307-311 (1959). ^b MIC values were determined on 1:1 (w/w) mixtures of amoxicillin and BL-P2013 or CP-45899, and 2:1 (w/w) mixtures of amoxicillin and clavulanic acid.

3 to the 2 β -(chloromethyl)penam 4 was carried out with benzoyl chloride and quinoline in refluxing dioxane. The use of quinoline is important in this step, since it appears to direct the rearrangement predominantly toward the penam 4 rather than toward the 3-chloro-3-methylcepham. Attempted catalytic reduction of 4 over 30% palladium on Celite to remove the 6-bromo and the *p*-nitrobenzyl groups was unsuccessful. When the sulfide was first converted to the sulfoxide 5 by oxidation with *m*-chloroperoxybenzoic acid, however, the reduction was successful. Subsequent oxidation with potassium permanganate in water afforded the sulfone 7 (BL-P2013), which was isolated as the potassium salt. Alternatively, the sulfide 4 could be oxidized to the sulfone 6 by treatment with potassium permanganate and 30% hydrogen peroxide in glacial acetic acid.⁸ Reduction of 6 with 30% palladium on Celite failed but was successful with 10% palladium on wide-pore carbon. The pivaloyloxymethyl ester 8 was

prepared in dimethylacetamide from pivaloyloxymethyl chloride and 7.

Biology. The minimum protective concentration (MPC) values reported in Table I illustrate the variation in inhibitory activity of several β -lactamase inhibitors against the β -lactamases of different bacterial species. It is interesting to note that the relative potencies found in the MPC values for BL-P2013, CP-45899, and clavulanic acid against the *Staphylococcus aureus* A9606 enzyme are not reflected in the amoxicillin protection data of Table II. In general, however, the ability to inhibit the β -lactamase enzyme (Table I) does appear to correlate with the ability to protect amoxicillin from the enzyme in vitro (Table II) and in vivo (Table III).

The data presented herein show that substitution of a chlorine on the β -methyl of penicillanic acid sulfone leads to significant changes in the β -lactamase inhibitory properties.

Experimental Section

Melting points were determined on a Fisher-Johns apparatus and are uncorrected. The IR spectra were recorded on a Beck-

(8) D. A. Johnson, C. A. Penetta, and D. E. Copper, *J. Org. Chem.*, 28, 1927 (1963).

Table III. Oral Mouse Protection Tests with a 1:1 Combination of Amoxicillin and BL-P2013 Using CP-45899 as a Control

| organism | challenge organisms ^a | oral PD ₅₀ , ^b mg/kg | | | | |
|-------------------------|----------------------------------|--|----------|----------|----------------------|----------------------|
| | | amoxicillin | BL-P2013 | CP-45899 | amox + BL-P2013, 1:1 | amox + CP-45899, 1:1 |
| <i>S. aureus</i> A9606 | 1 × 10 ⁹ | > 800 | > 200 | > 200 | 24 | 15 |
| <i>S. aureus</i> A15091 | 5 × 10 ⁸ | > 800 | > 200 | > 200 | 72 | 160 |
| <i>S. aureus</i> A20379 | 5 × 10 ⁸ | > 200 | > 200 | > 200 | 34 | 21 |
| <i>E. coli</i> A20649 | 6 × 10 ⁵ | > 200 | > 200 | > 200 | 25 | 90 |
| <i>E. coli</i> A21223 | 7 × 10 ⁵ | > 200 | > 200 | > 200 | 16 | 46 |

^a *S. aureus* and *E. coli* challenges were prepared in 2 and 4% mucin, respectively, and injected ip into 18–24 g Swiss-Webster (ICR Strain) mice. ^b Mice were treated orally at 0 and 2 h postchallenge. The experiments were terminated after 5 days, and the PD₅₀ was calculated by the method of Spearman and Karber, "Statistical Methods in Biological Assay", 2nd ed., Hafner Publishing Co., New York, 1964.

mann 5240 spectrophotometer using KBr pellets; NMR spectra were obtained on a Varian HA-100 spectrophotometer using (Me)₄Si as an internal standard. The IR and NMR spectra were consistent with the assigned structure.

6 α -Bromopenicillanic Acid 1-Oxide (2). To a suspension of 300 g (0.75 mol) of *N,N'*-dibenzylethylenediammonium 6 α -bromopenicillanate in 3 L of MeCl₂ at 5 °C was added dropwise with vigorous stirring 130 mL of concentrated HCl. The mixture was stirred at 5 °C for 2 h. The mixture was filtered through Celite and the filtrate was washed with 2 × 500 mL of H₂O. The MeCl₂ solution was dried over anhydrous Na₂SO₄, and the volume was adjusted to 750 mL by evaporating at 15 mm (32 °C). The solution was cooled to 5 °C and treated dropwise with 130 mL of 40% peroxyacetic acid, maintaining the temperature at 5–12 °C. After the addition was complete, the mixture was stirred at 5 °C for 2 h, and the sulfoxide (2) was collected by filtration and washed with cold H₂O and finally with MeCl₂. After the solution was dried in vacuo over P₂O₅, 126 g (57%) was obtained: mp 129 °C; IR (KBr) 1795 (s), 1740 (s) 1000 (m) cm⁻¹; ¹H NMR (Me₂SO, D₂O) δ 1.6 and 1.25 (2 s, 3, 3, 2 CH₃), 4.38 (s, 1, C₃ H), 5.15 (d, 1, C₅ H), 5.55 (d, 1, C₆ H). Anal. (C₈H₉BrNO₄S) C, H, N.

***p*-Nitrobenzyl 6 α -Bromopenicillanate 1-Oxide (3).** To a solution of 12 g (0.04 mol) of 6 α -bromopenicillanic acid 1-oxide in 100 mL of acetone was added 7.5 g (0.041 mol) of potassium 2-ethylhexanoate. The salt was collected by filtration, washed with cold acetone, and air-dried to yield a total of 10 g. The crystalline potassium salt was dissolved in 75 mL of DMAC, and 7.8 g (0.04 mol) of *p*-nitrobenzyl bromide was added. The solution was stirred at 23 °C for 24 h. The mixture was diluted with 500 mL of H₂O and extracted with EtOAc. The EtOAc layer was washed four times with H₂O and dried over anhydrous magnesium sulfate. The solvent was evaporated at 35 °C (15 mm) to an oil, which crystallized. The light tan crystals of 3 were slurried with ether and collected by filtration to yield 9 g (70%): mp 124–125 °C dec; IR (KBr) 1800 (s), 1740 (s), 1610 (w), 1520 (s), 1450 (m), 1350 (s), 1060 (m), 750 (m) cm⁻¹; ¹H NMR (Me₂SO) δ 1.22 (s, 3, CH₃), 1.6 (s, 3, CH₃), 4.67 (s, 1, C₃ H), 5.2 (d, 1, C₅ H), 4.67 (s, 1, C₃ H), 5.45 (s, 2, CH₂), 5.68 (d, 1, C₆ H), 7.5–8.5 (m, 4, aromatic). Anal. (C₁₅H₁₆BrN₂O₆S) C, H, N: calcd, 652; found, 6.98.

***p*-Nitrobenzyl 2 β -(Chloromethyl)-2 α -methyl-6 α -bromopenam-3 α -carboxylate (4).** A solution of 5 g (0.012 mol) of *p*-nitrobenzyl 6 α -bromopenicillanate 1-oxide (3) in 120 mL of anhydrous dioxane was heated at reflux under nitrogen for 4 h with 1.5 g (0.012 mol) of quinoline and 1.6 g (0.012 mol) of benzoyl chloride. The solution was diluted with 600 mL of H₂O and extracted with EtOAc. The EtOAc extract was washed with 5% NaHCO₃ solution, 5% H₃PO₄ solution, and finally with H₂O. The organic layer was dried over anhydrous MgSO₄ and evaporated to an oil at 35 °C (15 mm). The oil crystallized and was collected and washed with ether and finally with cold toluene to yield 3.5 g (65%): mp 130–135 °C dec; IR (KBr) 1792 (s), 1740 (s), 1610 (w), 1520 (s), 1353 (s), 1280 (m), 1025 (w), 990 (w), 750 (w) cm⁻¹; ¹H NMR (Me₂SO) δ 1.45 (s, 3, CH₃), 3.5–4.3 (m, 2, CH₂Cl), 5.05 (s, 1, C₃ H), 5.45 (s, 2, CH₂), 5.5 (d, 1, C₅ H), 5.62 (d, 1, C₆ H), 7.5–8.5 (m, 4, aromatic H). Anal. (C₁₅H₁₆BrClN₂O₄S) C, H, N.

***p*-Nitrobenzyl 2 β -(Chloromethyl)-2 α -methyl-6 α -bromopenam-3 α -carboxylate 1-Oxide (5).** A solution of 1 g (0.0022 mol) of *p*-nitrobenzyl 2 β -(chloromethyl)-2 α -methyl-6 α -bromopenam-3 α -carboxylate (4) dissolved in 50 mL of MeCl₂ was stirred with 473 mg (0.0022 mol) of *m*-chloroperoxybenzoic acid. The solution was stirred at 23 °C for 3 h. The MeCl₂ was evaporated to 20 mL at 15 mm and 33 °C, and the concentrated solution was diluted with 50 mL of heptane ("Skellysolve B"). The solvent was decanted, and the residue was slurried with ether; 5 soon crystallized to yield 250 mg (24%): mp 136–137 °C dec; IR (KBr) 1800 (s), 1760 (s), 1520 (s), 1350 (s), 1200 (s), 1050 (m), 830 (w), 740 (w) cm⁻¹; ¹H NMR (Me₂SO) δ 1.32 (s, 3, CH₃), 3.8–4.5 (m, 2, CH₂Cl), 4.97 (s, 1, C₃ H), 5.25 (d, 1, C₅ H), 5.45 (s, 2, CH₂O), 5.6 (d, 1, C₆ H), 7.8–8.5 (m, 4, aromatic). Anal. (C₁₅H₁₄BrClN₂O₆S) H, N; C: calcd, 38.68; found, 39.14.

***p*-Nitrobenzyl 2 β -(Chloromethyl)-2 α -methyl-6 α -bromopenam-3 α -carboxylate 1,1-Dioxide (6).** To a solution of 43.5 g (0.097 mol) of the sulfide 4 in 2 L of glacial acetic acid was added a solution of 34 g (0.215 mol) of potassium permanganate in 500 mL of H₂O. The mixture was treated dropwise with 30% H₂O₂ until the solution became clear. The solution was poured into 3 L of H₂O and stored for 1 h at 22 °C. The crystalline sulfone was collected, washed with H₂O, and dried in vacuo over P₂O₅ to yield 40.5 g (87%): mp 130–132 °C; IR (KBr) 1805 (s), 1760 (s), 1345, 1520 (s), 1335, 1440 (s) cm⁻¹; ¹H NMR (CDCl₃) δ 1.5 (s, 3, CH₃), 3.6–4.1 (m, 2, CH₂Cl), 4.75 (d, 1, C₅ H), 4.8 (s, 1, C₃ H), 5.2 (d, 1, C₆ H), 5.4 (s, 2, CH₂O), 7.4–8.5 (m, 4, aromatic). Anal. (C₁₅H₁₆BrClO₇S) C, H, N.

Potassium 2 β -(Chloromethyl)-2 α -methylpenam-3 α -carboxylate 1,1-Dioxide (7, BL-P2013). A. To a solution of 7 g (0.015 mol) of *p*-nitrobenzyl 2 β -(chloromethyl)-2 α -methyl-6 α -bromopenam-3 α -carboxylate 1-oxide (5) in 150 mL of EtOAc was added a suspension of 4 g of 30% palladium on Celite and 2.8 g of NaHCO₃ in 150 mL of H₂O. The mixture was hydrogenated for 3 h at 50 psi. The catalyst was removed by filtration, and the aqueous layer was separated and treated with 1.5 g of KMnO₄ in 50 mL of H₂O. The mixture was filtered, and the filtrate was adjusted to pH 2 with concentrated HCl. The solution was lyophilized to give a white amorphous powder. The solid was extracted with EtOAc, evaporated to a volume of 20 mL, and diluted with 100 mL of heptane ("Skellysolve B"). Colorless, hygroscopic, solid 2 β -(chloromethyl)-2 α -methylpenam-3 α -carboxylic acid sulfone was collected. The acid was dissolved in acetone and treated with solid potassium 2-ethylhexanoate. A crystalline white salt precipitated to give, after filtration, 170 mg, mp 140 °C dec.

B. To a solution of 8 g (0.017 mol) of *p*-nitrobenzyl 2 β -(chloromethyl)-2 α -methyl-6 α -bromopenam-3 α -carboxylate 1,1-dioxide (6) in 100 mL of EtOAc was added a suspension of 4 g of 10% palladium on wide-pore carbon (Engelhardt) and 4 g of NaHCO₃ in 150 mL of H₂O. The mixture was hydrogenated for 4 h at 50 psi at 22 °C. The catalyst was removed by filtration, and the aqueous layer was separated. The solution was adjusted with concentrated HCl dropwise to pH 1.5 and saturated with sodium sulfate. The mixture was extracted with EtOAc. The

EtOAc was washed with brine and dried over anhydrous Na_2SO_4 . The solvent was evaporated at 32 °C (15 mm) to an oily residue, which was dissolved in 30 mL of acetone and 30 mL of ether. The solution was treated with solid potassium 2-ethylhexanoate until the solution was pH 6. The precipitate was collected and dried to yield 3.9 g (76%): IR (KBr) 1790 (s), 1700 (m), 1620 (s), 1460 (m), 1370 (s), 1310 (s), 1200 (s), 1140 (s), 955 (m), 740 (m) cm^{-1} ; ^1H NMR (D_2O) δ 1.68 (s, 3, CH_3), 3.2-3.9 (m, 2, C_6H_2), 4.0-4.4 (m, 2, CH_2Cl), 4.3 (s, 1, C_3H), 5.02 (d, 1, C_5H). Anal. ($\text{C}_8\text{H}_7\text{ClKNO}_5\text{S}\cdot 2\text{H}_2\text{O}$) C, N; H: calcd, 3.24; found, 3.69.

Pivaloyloxymethyl 2 β -(Chloromethyl)-2 α -methyl-3 α -carboxylate 1,1-Dioxide (8). To a solution of 1 g (0.0031 mol) of 7 dissolved in 15 mL of DMAC was added 470 mg (0.0031 mol) of pivaloyloxymethyl chloride. The solution was stirred for 24 h. The mixture was filtered, poured into 120 mL of H_2O , and extracted with EtOAc. The EtOAc was washed 9 times with H_2O and dried over anhydrous MgSO_4 . The EtOAc was evaporated at 35 °C (15 mm) to an oil, which was chromatographed in SiliCAR CC-7 (80 MeCl_2 /20 EtOAc). The amorphous solid obtained was

dissolved in 50 mL of Skellysolve B and stored at 15 °C for 10 h. The crystals were collected by filtration, affording a total of 239 mg: mp 94-95 °C; IR (KBr) 1800 (s), 1770 (s), 1320, 1125 (w) cm^{-1} ; ^1H NMR (CDCl_3) δ 1.25 [s, 1, (CH_3)₃C], 1.65 (s, 1, CH_3), 3.3-3.75 (m, 2, C_6H_2), 3.75-4.2 (m, 2, CH_2Cl), 4.6 (s, 1, C_3H), 4.6-4.8 (m, 1, C_5H), 5.7-6.0 (m, 2, OCH_2O). Anal. ($\text{C}_{13}\text{H}_{20}\text{ClNO}_5\text{S}$) C, H, N.

Acknowledgment. We thank the Analytical Research and Development Department (and, in particular, Elizabeth Ragan, David Whitehead, and Victoria Gross) for the elemental analyses and NMR and IR spectra. We also thank the Microbiological Research Department for supplying the microbiological data (and, in particular, Dr. Felix Leitner, Dr. David Chisholm, Douglas Behr, Robert Buck, Jacqueline Randolph, Teresa Pursiano, and Dr. Martin Misiek).

Interaction of Aromatic Dyes with the Coenzyme A Binding Site of Choline Acetyltransferase

Henry G. Mautner,* Robert E. Merrill, Stephen F. Currier, and Gregory Harvey

Department of Biochemistry and Pharmacology, Tufts University School of Medicine, Boston, Massachusetts 02111.
Received March 16, 1981

The interaction of a series of aromatic dyes with the coenzyme A binding site of choline acetyltransferase was studied. Several of the dyes were very potent inhibitors of the enzyme. With few exceptions, inhibition was competitive with respect to acetylcoenzyme A and noncompetitive with respect to choline. It appears likely that inhibition by dyes such as Reactive Blue 2 (Cibacron Blue F3GA) or Congo Red, as in the case of coenzyme A interactions, involves hydrophobic bonding, as well as a coulombic interaction with an arginine residue.

Considering that chemotherapy was based on Ehrlich's studies of the specificity of dye interactions, it seems surprising that, even though studies of the structure-action relationships of aromatic dyes led to the development of many medicinally useful agents, the molecular basis of the specificity of dye interactions was studied little until recent years. Glazer¹ noted that aromatic dyes with no obvious structural relationships to normally attached ligands can bind very tightly to globular proteins, predominantly to areas overlapping binding sites for coenzymes or substrates. Crystallographic studies of dehydrogenase-dye complexes have confirmed this.²⁻⁴ The specificity of the interaction of aromatic dyes with the coenzyme-binding sites of certain enzymes is confirmed by the utility of solid supports to which aromatic dyes are attached in the chromatographic purification of such biopolymers. While numerous dyes have been utilized for this purpose, columns carrying Cibacron Blue F3GA have found particularly wide use.⁵

The present study is concerned with the specificity of the interaction of aromatic dyes with choline acetyltransferase (ChA), the enzyme catalyzing the reaction of choline with acetyl-CoA to form acetylcholine, a molecule

essential for the functions of the nervous system.

Roskoski et al.⁶ noted that Blue Dextran columns could be used for the purification of ChA. This procedure was improved by Hersh et al.,⁷ who used acetyl-CoA as eluant.

Thompson and his co-workers⁸ had claimed that the inhibitory action of Reactive Blue 2 (Cibacron Blue F3GA), the chromophore of Blue Dextran, was based on the structural resemblance of this dye to the adenosyl diphosphoryl portion of coenzymes; however, several workers⁹⁻¹¹ questioned the assumption that dye-binding sites and coenzyme-binding sites were very similar. During a study of the abilities of a series of derivatives of CoA to serve as inhibitors of ChA, we noted that not only Reactive Blue 2 but the much simpler molecule 1-anilino-naphthalene-8-sulfonic acid (1,8-ANS) appeared to be a competitive inhibitor of acetyl-CoA.^{12,13} These findings

- (1) A. N. Glazer, *Proc. Natl. Acad. Sci. U.S.A.*, **65**, 1057 (1970).
- (2) P. M. Wassarman and P. J. Lentz, *J. Mol. Biol.*, **60**, 509 (1971).
- (3) R. H. Einarsson, H. Eklund, E. Zeppezauer, T. Boiwe, and C. I. Bränden, *Eur. J. Biochem.*, **49**, 41 (1974).
- (4) J. F. Biellmann, J. P. Samama, C. I. Bränden, and H. Eklund, *Eur. J. Biochem.*, **102**, 107 (1979).
- (5) For a review, see E. Stellwagen, *Acc. Chem. Res.*, **10**, 92 (1977).

- (6) R. J. Roskoski, C. T. Lim, and L. M. Roskoski, *Biochemistry*, **14**, 5105 (1975).
- (7) L. B. Hersh, B. Coe, and L. Casey, *J. Neurochem.*, **30**, 1077 (1978).
- (8) S. T. Thompson, K. H. Cass, and E. Stellwagen, *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 669 (1975).
- (9) J. E. Wilson, *Biochem. Biophys. Res. Commun.*, **72**, 816 (1976).
- (10) A. R. Ashton and G. M. Polya, *Biochem. J.*, **175**, 501 (1978).
- (11) R. E. Barden, P. L. Darke, R. A. Deems, and E. A. Dennis, *Biochemistry*, **19**, 1621 (1980).
- (12) S. F. Currier and H. G. Mautner, *Biochemistry*, **16**, 1944 (1977).
- (13) H. G. Mautner, *CRC Crit. Rev. Biochem.*, **4**, 341 (1977).