1.0 mL of AcOH and spin-evaporated in vacuo. The residue was dissolved in 50 mL of H_2O and applied to a column of Amberlite XAD-2 nonionic polymeric absorbant (200 g, 3.5 × 31 cm). The column was eluted with H_2O 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 C_6H_6 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 (Me_2SO-d_6) δ 7.77 (s, 1 H, C-8), 6.44 (br s, 2 H, NH_2), 5.31 (s, 2 H, NCH_2O), 4.38 (br s, 1 H, NH), 3.6–3.2 (m, 5 H, $2CCH_2N$ + OH), 2.7–2.4 (m, 4 H, $2CCH_2O$). Anal. ($C_{10}H_{16}N_6O_3$) 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 H_2O 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 Et_2O, 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. ($C_{10}H_{14}N_6O_3$) 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 IC_{50} 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 2β-(Chloromethyl)-2α-methylpenam-3α-carboxylic Acid 1,1-Dioxide

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Received June 15, 1981

Potassium 2β-(chloromethyl)-2α-methylpenam-3α-carboxylate 1,1-dioxide (BL-P2013) and its pivaloyloxymethyl ester were prepared by the conversion of 6-aminopenicillanic acid to *p*-nitrobenzyl 6α-bromo-2,2-dimethylpenam-3α-carboxylate 1-oxide, which was rearranged with benzoyl chloride and quinoline to *p*-nitrobenzyl 6α-bromo-2β-(chloromethyl)-2α-methylpenam-3α-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 acid[1] 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] 6β-bromopenicillanic acid,[3] 6α-chloropenicillanic acid sulfone,[4] and various 6β-(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, 2β-(chloromethyl)-2α-methylpenam-3α-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 vitro and in vivo test systems.

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

compd	min protective concn,[a] μg/mL		
	K.p.	*S.a.*	*B.f.*
methicillin sulfone	125	>100	
6β-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.

Chemistry. 6α-Bromopenicillanic acid (**1**) was prepared from 6β-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 peroxyacetic 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

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Scheme I

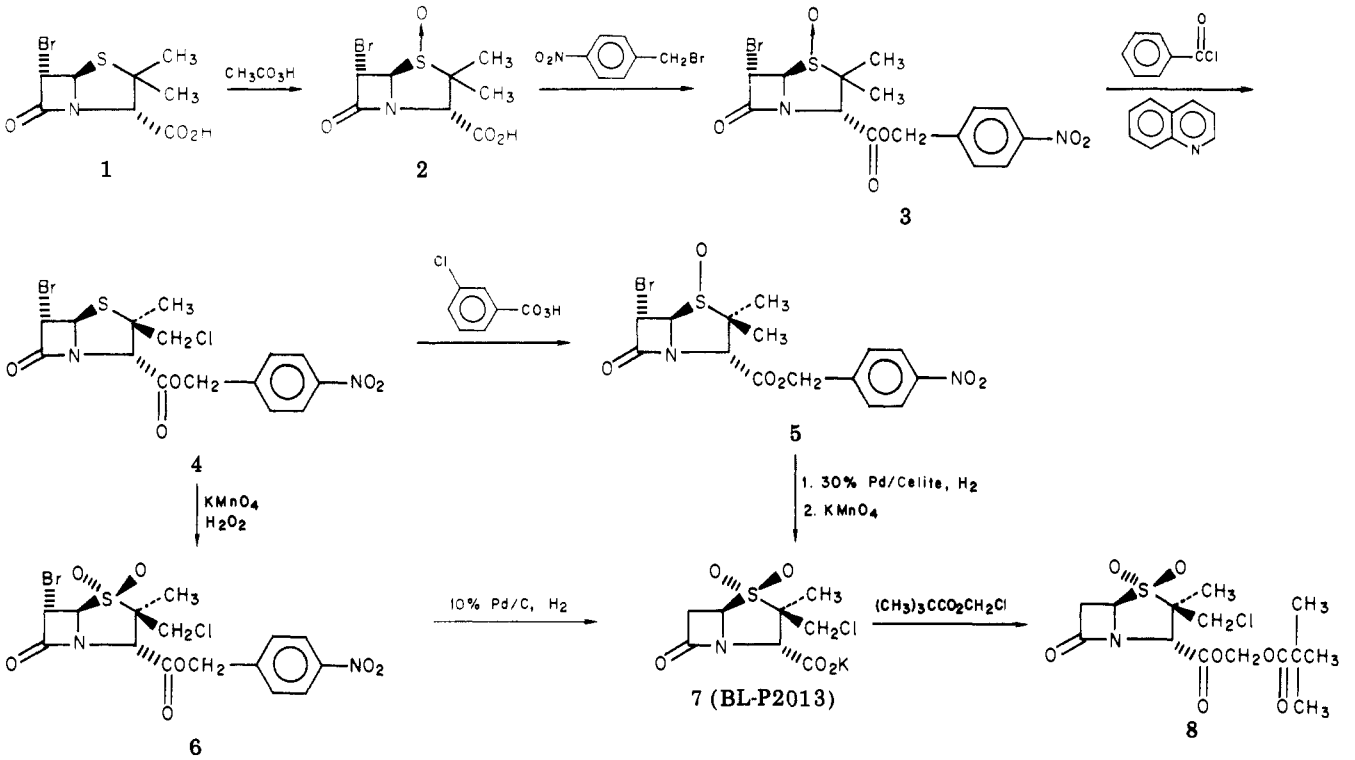

Table II. Synergistic Combinations of Amoxicillin with Various β-Lactamase Inhibitors

	min inhibitory concn,[a] μg/mL						
organism	amoxicillin	amoxicillin + BL-P2013, 1:1[b]	BL-P2013	CP-45899	amoxicillin + CP-45899, 1:1[b]	clavulanic acid	amoxicillin + clavulanic acid, 2:1[b]
S. aureus A9606	>125	1:1	125	>125	2:2	32	2:1
S. aureus A15091	>125	1:1	125	>125	2:2	32	2:1
S. aureus A20309	125	2:2	125	>125	4:4	32	2:1
E. coli A9675	63	2:2	>125	125	4:4	32	2:1
E. coli A21223	125	2:2	>125	>125	4:4	32	8:4

[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 2β-(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-

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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
S. aureus A9606	1×10^9	>800	>200	>200	24	15
S. aureus A15091	5×10^8	>800	>200	>200	72	160
S. aureus A20379	5×10^8	>200	>200	>200	34	21
E. coli A20649	6×10^5	>200	>200	>200	25	90
E. coli 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 PD_{50} 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 consistant with the assigned structure.

6α-Bromopenicillanic Acid 1-Oxide (2). To a suspension of 300 g (0.75 mol) of *N,N'*-dibenzylethylenediammonium 6α-bromopenicillanate in 3 L of $MeCl_2$ 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 H_2O. The $MeCl_2$ solution was dried over anhydrous Na_2SO_4, 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 H_2O and finally with $MeCl_2$. After the solution was dried in vacuo over P_2O_5, 126 g (57%) was obtained: mp 129 °C; IR (KBr) 1795 (s), 1740 (s) 1000 (m) cm^{-1}; ^1H NMR (Me_2SO, D_2O) δ 1.6 and 1.25 (2 s, 3, 3, 2 CH_3), 4.38 (s, 1, C_3 H), 5.15 (d, 1, C_5 H), 5.55 (d, 1, C_6 H). Anal. ($C_8H_9BrNO_4S$) C, H, N.

***p*-Nitrobenzyl 6α-Bromopenicillanate 1-Oxide (3).** To a solution of 12 g (0.04 mol) of 6α-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 H_2O and extracted with EtOAc. The EtOAc layer was washed four times with H_2O 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 (Me_2SO) δ 1.22 (s, 3, CH_3), 1.6 (s, 3, CH_3), 4.67 (s, 1, C_3 H), 5.2 (d, 1, C_5 H), 4.67 (s, 1, C_3 H), 5.45 (s, 2, CH_2), 5.68 (d, 1, C_6 H), 7.5–8.5 (m, 4, aromatic). Anal. ($C_{15}H_{15}BrN_2O_6S$) C, H; N: calcd, 652; found, 6.98.

***p*-Nitrobenzyl 2β-(Chloromethyl)-2α-methyl-6α-bromopenem-3α-carboxylate (4).** A solution of 5 g (0.012 mol) of *p*-nitrobenzyl 6α-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 H_2O and extracted with EtOAc. The EtOAc extract was washed with 5% $NaHCO_3$ solution, 5% H_3PO_4 solution, and finally with H_2O. The organic layer was dried over anhydrous $MgSO_4$ 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 (Me_2SO) δ 1.45 (s, 3, CH_3), 3.5–4.3 (m, 2, CH_2Cl), 5.05 (s, 1, C_3 H), 5.45 (s, 2, CH_2), 5.5 (d, 1, C_5 H), 5.62 (d, 1, C_6 H), 7.5–8.5 (m, 4, aromatic H). Anal. ($C_{15}H_{15}BrClN_2O_4S$) C, H, N.

***p*-Nitrobenzyl 2β-(Chloromethyl)-2α-methyl-6α-bromopenem-3α-carboxylate 1-Oxide (5).** A solution of 1 g (0.0022 mol) of *p*-nitrobenzyl 2β-(chloromethyl)-2α-methyl-6α-bromopenam-3α-carboxylate (4) dissolved in 50 mL of $MeCl_2$ was stirred with 473 mg (0.0022 mol) of *m*-chloroperoxybenzoic acid. The solution was stirred at 23 °C for 3 h. The $MeCl_2$ 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 (Me_2SO) δ 1.32 (s, 3, CH_3), 3.8–4.5 (m, 2, CH_2Cl), 4.97 (s, 1, C_3 H), 5.25 (d, 1, C_5 H), 5.45 (s, 2, CH_2O), 5.6 (d, 1, C_6 H), 7.8–8.5 (m, 4, aromatic). Anal. ($C_{15}H_{14}BrClN_2O_6S$) H, N; C: calcd, 38.68; found, 39.14.

***p*-Nitrobenzyl 2β-(Chloromethyl)-2α-methyl-6α-bromopenam-3α-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 H_2O. The mixture was treated dropwise with 30% H_2O_2 until the solution became clear. The solution was poured into 3 L of H_2O and stored for 1 h at 22 °C. The crystalline sulfone was collected, washed with H_2O, and dried in vacuo over P_2O_5 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 ($CDCl_3$) δ 1.5 (s, 3, CH_3), 3.6–4.1 (m, 2, CH_2Cl), 4.75 (d, 1, C_5 H), 4.8 (s, 1, C_3 H), 5.2 (d, 1, C_6 H), 5.4 (s, 2, CH_2O), 7.4–8.5 (m, 4, aromatic). Anal. ($C_{15}H_{19}BrClO_7S$) C, H, N.

Potassium 2β-(Chloromethyl)-2α-methylpenam-3α-carboxylate 1,1-Dioxide (7, BL-P2013). A. To a solution of 7 g (0.015 mol) of *p*-nitrobenzyl 2β-(chloromethyl)-2α-methyl-6α-bromopenam-3α-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 $NaHCO_3$ in 150 mL of H_2O. 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 $KMnO_4$ in 50 mL of H_2O. 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 2β-(chloromethyl)-2α-methylpenem-3α-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 2β-(chloromethyl)-2α-methyl-6α-bromopenam-3α-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 $NaHCO_3$ in 150 mL of H_2O. 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 wad 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_3 H), 5.02 (d, 1, C_5 H). Anal. ($C_8H_7ClKNO_5S\cdot 2H_2O$) C, N; H: calcd, 3.24; found, 3.69.

Pivaloyloxymethyl 2β-(Chloromethyl)-2α-methyl-3α-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 Sili*CAR* 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_3 H), 4.6–4.8 (m, 1, C_5 H), 5.7–6.0 (m, 2, OCH_2O). Anal. ($C_{13}H_{20}ClNO_5S$) 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

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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. Glazer[1] 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-workers[8] 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 workers[9-11] 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-anilinonaphthalene-8-sulfonic acid (1,8-ANS) appeared to be a competitive inhibitor of acetyl-CoA.[12,13] These findings

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