Chloride. Compound VI (3.0 g, 6.35 mmol) was dissolved in AcOEt (50 mL) containing 10% dry HCl and stirred for 3 h. Cold Et_2O (70 mL) was added, and the precipitated material, ethyl 4-methyl-1-(N^{ω}-nitro-L-arginyl)piperidinecarboxylate hydrochloride (VII), was centrifuged and washed with Et_2O (2 × 20 mL) by successive centrifugation and decantation, the precipitation being well suspended in each wash by vortex mixing. The product was dried in vacuo. To a mixture of VII (2.50 g, 6.1 mmol) and Et_3N (2.1 g, 20 mmol) in CH_2Cl_2 (30 mL) was added 7-methoxy-2-naphthalenesulfonyl chloride (1.94 g, 7.5 mmol) with stirring at 0–5 °C. After 4 h, the mixture was washed with H_2O, dried (Na_2SO_4), and evaporated. The oily residue was chromatographed on a silica gel column eluting with $CHCl_3$–MeOH (97:3). Evaporation of the eluate gave ethyl 1-[N^{α}-(7-methoxy-2-naphthalenesulfonyl)-N^{ω}-nitroarginyl]-4-methyl-2-piperidinecarboxylate (VIII; 3.2 g, 89%) as an amorphous solid. Anal. ($C_{26}H_{36}N_6O_8S$) C, H, N.

C. Removal of the NO_2 Group. Compound VIII (3.20 g, 5.38 mmol) was dissolved in EtOH (20 mL) and AcOH (2 mL), and Pd black (0.50 g) was added. H_2 was bubbled into the mixture for 30 h at room temperature. After we filtered off the catalyst, the filtrate was evaporated to give a viscous oily product. Reprecipitation from EtOH–Et_2O gave ethyl 1-[N^{α}-(7-methoxy-2-naphthalenesulfonyl)-L-arginyl]-4-methyl-2-piperidinecarboxylate (IX; 2.44 g, 83%) as an amorphous solid. For analysis of the product, a portion of the product was converted to the flavianate, mp 188–189 °C. Anal. ($C_{26}H_{37}N_5O_6S \cdot C_{10}H_6N_2O_8S$) C, H, N.

D. Hydrolysis of IX. The title compound (**49**) as an amorphous solid was prepared in manner similar to that for method B: IR (KBr) 3250 (br), 1625 cm^{-1}. Anal. ($C_{24}H_{33}N_5O_6S$) C, H, N.

Inhibition Studies of the Clotting Activity of Thrombin. The method has been described in the preceding paper.[1] The clotting time was measured at 25 °C in the reaction mixture, at a final volume of 1.0 mL containing 0.096% fibrinogen.

Acknowledgment. The authors thank A. Maruyama and Mrs. K. Sugano for their technical assistance and Dr. S. Hattori, General Manager of Biosciences Laboratory, Central Research Laboratories, Mitsubishi Chemical Industries Limited, for his valuable advice and encouragement throughout the work. We are also indebted to members in Systems Engineering Laboratory, Central Research Laboratories, Mitsubishi Chemical Industries Limited, for the elemental analyses.

Analogues of Chloramphenicol: Circular Dichroism Spectra, Inhibition of Ribosomal Peptidyltransferase, and Possible Mechanism of Action

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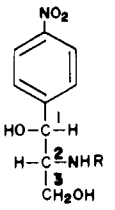
Circular dichroism spectra of a series of chloramphenicol derivatives **1a–r** were measured in water at pH 7. Compounds **1a–o** exhibit two positive Cotton effects at 310–340 and 240–260 nm, respectively, and a weaker negative Cotton effect at 280–300 nm. In analogues **1c**, 11, and **1m** there is only a minimum between the two positive Cotton effects. Derivatives **1p–r** possess a strong negative Cotton effect at ca. 280 nm. Compounds **1a–r** were examined as inhibitors of the puromycin reaction with *Escherichia coli* 70S ribosome–poly(U)–*N*-AcPhe-tRNA complex. Analogues 11, **1n**, **1o**, and **1q** are potent competitive inhibitors of puromycin comparable to or better than chloramphenicol (**1b**). Compounds **1k** and **1m** are less active, whereas **1d–g** and **1j** are only moderately effective. The rest of the analogues have marginal or no activity. The results are compared with previous biological data and discussed in terms of a retro-inverso relationship of chloramphenicol (**1b**) to the aminoacyl moiety of puromycin (aminoacyl-tRNA) and to a hypothetical transition state of peptide bond formation.

The antibiotic chloramphenicol (**1b**) is a powerful inhibitor of protein synthesis in bacteria.[1] Extensive studies have established that **1b** inhibits peptide bond synthesis catalyzed by peptidyltransferase at, or close to, the ribosomal A site.[2] Although it has been inferred from these investigations that **1b** is an analogue of aminoacyl-tRNA or puromycin, attempts to relate its structure to various portions of the 3′ terminus of aminoacyl-tRNA (puromycin) have not yet met with success. Thus, X-ray diffraction[3] and NMR[4] studies of **1b** led to postulation of a structure designated "curled" conformation (**2**) stabilized by hydrogen bonding which is probably maintained during binding of **1b** to ribosomes.[5] Another study[6] has shown that conformation **2** is stable in solution even in the absence of hydrogen bonding. According to one proposal,[4] conformer **2** can resemble uridine 5′-phosphate (UMP). There is, however, little to suggest that UMP plays a role in protein synthesis. This concept was later extended[7] to include pyrimidine 5′-nucleotides, in general, because of a similarity of chloramphenicol action to that of the cytosine-containing antibiotics gougerotin and blasticidin.



1a: R = H
1b: R = Cl_2CHCO
1c: R = CHO
1d: R = CH_3CO
1e: R = C_2H_5CO
1f: R = n-C_3H_7CO
1g: R =  CO
1h: R =  CO
1i: R = $HOCH_2CO$
1j: R = FCH_2CO
1k: R = $ClCH_2CO$
1l: R = $CNCH_2CO$
1m: R = N_3CH_2CO
1n: R = Br_2CHCO
1o: R = Cl_3CCO
1p: R = CO
1q: R = CH_3SO_2
1r: R = $ClCH_2PO(OMe)$

(1) O. Pongs, "Drug Action at the Molecular Level", G. C. K. Roberts, Ed., University Park Press, Baltimore, 1977, p 190.
(2) D. Vazquez, *FEBS Lett.*, **suppl. 40**, 63 (1974).
(3) J. D. Dunitz, *J. Am. Chem. Soc.*, **74**, 995 (1952).
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(6) T. M. Bustard, R. S. Egan, and T. J. Perun, *Tetrahedron*, **29**, 1961 (1973).
(7) C. Coutsogeorgopoulos, *Biochim. Biophys. Acta*, **129**, 214 (1966).

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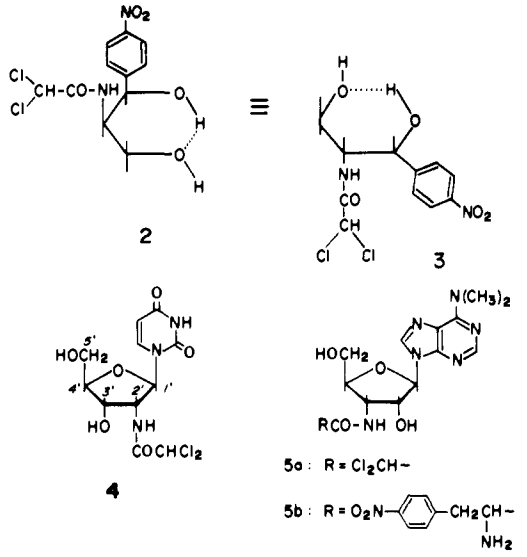
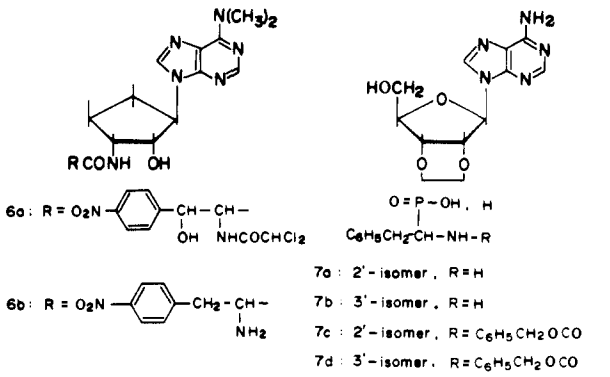


More recently, cytidine 5′-phosphate (CMP) was found to stimulate activity of "incomplete" donors of the peptide moiety at the ribosomal P site.[8] However, it is difficult to relate this activity to the fact that **1b** is essentially an A-site inhibitor.[1,2]

There is also little evidence to support the thesis that **1b** in conformation **2** is an analogue of 2′- or 3′-aminoacyl nucleosides (puromycin).[9-11] The suggestion was apparently based on findings that some aminoacyl analogues of **1b** are capable of inhibiting ribosome-catalyzed poly(Phe) synthesis.[7,9,11] However, it is clear from formula **3** that **1b** resembles rather an α-anomer of the corresponding 2′-aminoacyl nucleoside. It is, therefore, not surprising that attempts to relate **1b** to β-anomers of 2′-aminoacyl nucleosides[10,12] have also not been successful. Thus, a study of the inhibition of chloramphenicol binding to ribosomes with isomeric 2′- and 3′-aminoacyl nucleosides and their cytidylyl-3′–5′ derivatives has shown[12] that 3′-isomers are mostly better inhibitors than 2′-isomers. In addition, we have found (vide infra) that compound **4**, a suggested[10] "hybrid" of **1b** and puromycin, is devoid of biological activity. A similar "3′" derivative, **5a**, exhibited only a marginal effect.[9]

The proposal that **1b** can act as an analogue either of aminoacyl-[11] or peptidyl-tRNA[13] or puromycin was also rejected[9] on the basis of model studies. Thus, compounds **6a** and **6b** did not inhibit ribosome-catalyzed poly(U,-C)-directed poly(Phe) synthesis.[9] However, it is known[14] that conversion of **1b** to the corresponding 3-phenylserine derivatives abolished biological activity. In addition, more recent results have indicated that analogue **5b** is able to participate in ribosome-catalyzed peptide synthesis.[15] Therefore, it is not possible to exclude that **1b**, or at least a part of the molecule, is an analogue of the aromatic amino acid in puromycin.



Finally, it has been proposed[16-18] that the basis of chloramphenicol action is a covalent attachment of a radical derived from the cleavage of the C_1–H bond to a receptor site. These suggestions are at variance with the fact that **1b** is a reversible inhibitor[1] of protein synthesis.

Further progress in the investigation of a structure–activity relationship of **1b** can be expected by studying the physicochemical properties of a more extensive series of analogues and a correlation of the obtained results with biological data from both in vivo and simplified cell-free systems. The results of such a study—circular dichroism (CD) spectra and inhibition of the ribosome-catalyzed puromycin reaction—are the subject of this article.

CD Spectra. To our knowledge, only incomplete CD data are available[19] for chloramphenicol (**1b**), its stereoisomers, and the parent base **1a**. Thus, the CD spectra of **1b** in methanol revealed a positive Cotton band at longer wavelength (ca. 340 nm) which was assigned ^1L_b and another positive band at ca. 260 nm identified as ^1L_a. Little attention was paid to the region between 280 and 310 nm, although it includes the UV maximum of **1b**.

We have measured the CD spectra of compounds **1a–r** in water at pH 7 throughout the region of 230–400 nm. Two positive Cotton effects at 310–340 and 240–260 nm, respectively, are apparently similar to those observed previously[19] in methanol. It should be noted that molar ellipticity of the long-wavelength band is fairly constant (Table I) throughout the series **1a–r** ([θ] 500–1000) with the exception of the *N*-furoyl derivative **1p**, which contains an additional strong chromophore and where both positive Cotton effects are virtually missing. The positive band between 240 and 260 nm (presumably ^1L_a) is usually of higher ellipticity and it also varies more substantially within the series **1a–q**. A third, negative, Cotton band found in the region of the UV maximum of **1b** (280–310 nm, cf. Figure 1) is weak in chloramphenicol base **1a** and the corresponding *N*-acyl derivatives **1b–o**. Compounds **1c**, **11**, and **1m** (Table I) have only a minimum between both positive Cotton effects. The negative Cotton band is very strong in *N*-furoyl derivative **1p** and, more surprisingly, in methylsulfonyl and chloromethylphosphonyl analogues **1q** and **1r** ([θ] 2500). These pronounced differences may be attributed to the presence of an additional chromophore in **1p**, but such an explanation is obviously inadequate for **1q** and **1r**. It is also not likely that an inductive effect of substituents attached to the carbonyl (sulfonyl) group is responsible for the results. Thus, methylsulfonic acid is the strongest of all listed in Table I,

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(10) J. P. H. Verheyden, D. Wagner, and J. G. Moffatt, *J. Org. Chem.*, 36, 250 (1971).
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(12) R. Goldberg, D. Ringer, and S. Chládek, *Eur. J. Biochem.*, 81, 373 (1977).
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(14) C. W. Moersch, M. C. Rebstock, A. C. Moore, and D. P. Hylander, *J. Am. Chem. Soc.*, 74, 565 (1952).
(15) (a) M. Ariatti, personal communication; (b) H. J. Lee, K. L. L. Fong, and R. Vince, *J. Med. Chem.*, submitted.
(16) E. Kutter and H. Machleidt, *J. Med. Chem.*, 14, 931 (1971).
(17) C. Hansch, K. Nakamoto, M. Gorin, P. Denisevich, E. R. Garrett, S. M. Heman-Ackah, and C. H. Won, *J. Med. Chem.*, 8, 917 (1973).
(18) H.-D. Höltje and L. B. Kier, *J. Med. Chem.*, 17, 814 (1974).
(19) L. A. Mitscher, F. Kautz, and J. LaPidus, *Can. J. Chem.*, 47, 1957 (1969).

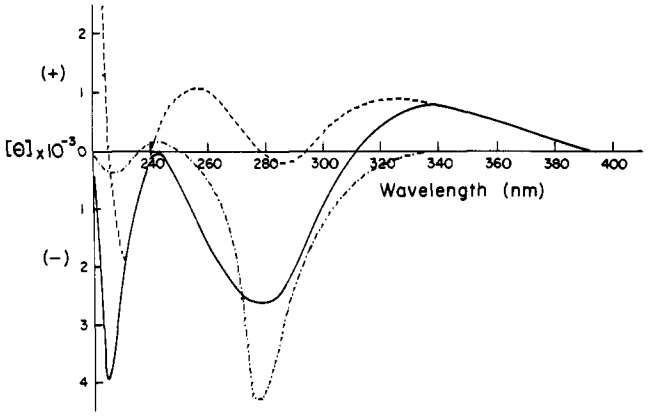
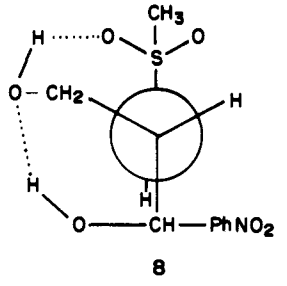


Figure 1. CD spectra of chloramphenicol (**1b**) and analogue **1p** and **1q** in water at pH 7: (- - -) **1b**; (—) **1q**; (-•-) **1p**.

but chloromethylphosphonic acid is weaker than dichloro- or trichloroacetic acid. In addition, despite that fact that the pK_a values of the parent carboxylic acids vary over a range of three orders of magnitude, these changes are reflected only to a very limited extent in both long-wavelength Cotton bands of analogues **1b–o**. Even more striking in this respect is the small difference between chloramphenicol base **1a**, presumably protonated at pH 7, and the *N*-acyl derivatives **1b–o**. This may indicate that factors such as perturbation of the aromatic π system by a through-space effect of a carbonyl group or possible *E,Z* isomerism of the amide are also of minor significance. Therefore, it can be argued that the more pronounced negative long-wavelength Cotton effect may simply reflect the differences in the conformation of **1q** and **1r** relative to the rest of the group. However, it appears unlikely that changes in the rotameric composition around the C_1–C_2 bond, which would adversely affect the hydrogen bonding (formula 2 or 3), would not also result in a decrease of the biological activity of **1q** which, incidentally, is very high (vide supra). On the other hand, an additional oxygen atom in **1q** can provide more extensive hydrogen-bonding stabilization (conformation 8) relative to **1b**, shown as a



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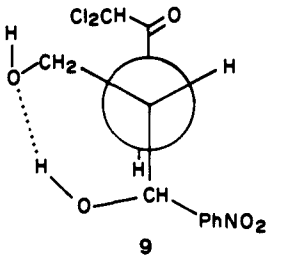

9

Newman projection, **9**, of structure **2** or **3**. It is possible that an increased rigidity of structure **8** can lead to an enhancement of the Cotton effect at 280 nm. Nevertheless, it is recognized that conformational studies of **1q** using methods complementary to CD spectra are needed to bring more information on this point.

Inhibition of Ribosomal Peptidyltransferase. Despite the fact that many chloramphenicol analogues modified in the acyl amide moiety were tested for biological activity,[17,20,21] less data are available on their examination in cell-free systems. Almost without exception, inhibition

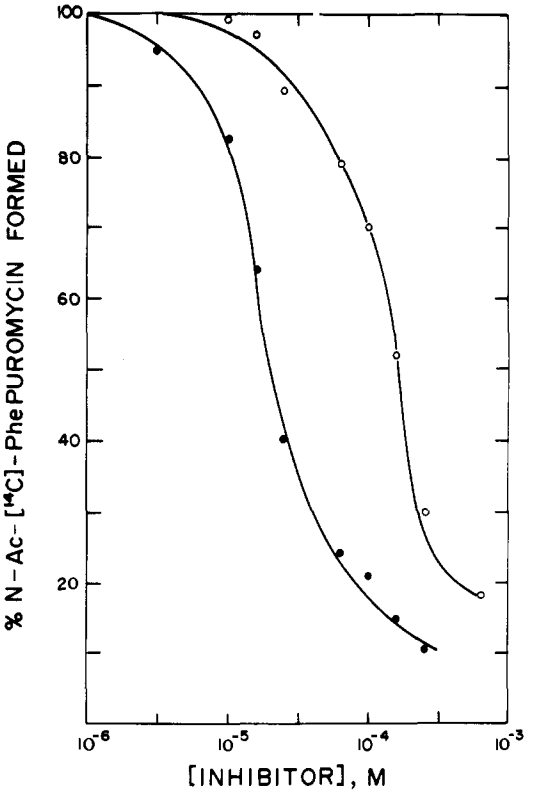

Figure 2. Inhibition of the puromycin reaction with chloramphenicol (**1b**) and methylsulfonyl analogue **1q**: (●) **1b**; (○) **1q**.

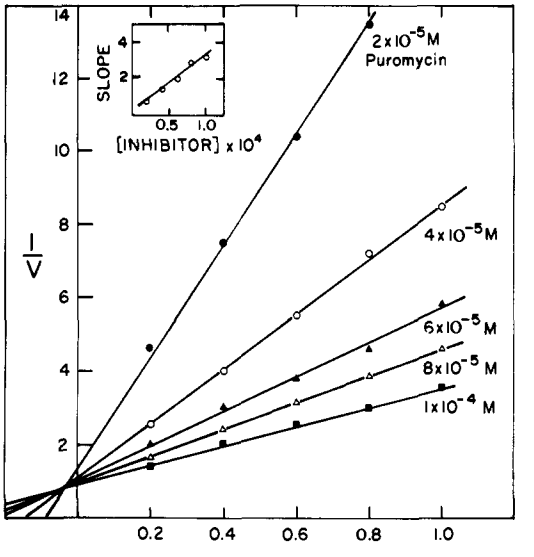

Figure 3. Dixon plot of the effect of methylsulfonyl derivative **1q** on the rate of *N*-acetyl-L-[^{14}C]phenylalanylpuromycin formation.

of poly(U,C)- or poly(U)-mediated ribosomal polypeptide synthesis was used in the latter studies,[7,9] whereas very limited attention was paid to inhibition of the puromycin reaction,[22] a system which makes possible synthesis of only a single peptide bond. Also, little comparative information is available on data obtained from in vivo studies and those from cell-free assays.[23] It was, therefore, of interest to

(20) F. E. Hahn, J. E. Hayes, C. L. Wiseman, Jr., H. E. Hopps, and J. E. Smadel, *Antibiot. Chemother.* **6**, 531 (1956).
(21) M. M. Shemyakin, M. N. Kolosov, M. M. Levitov, K. I. Germanova, M. G. Karapetyan, Yu. B. Shvetsov, and E. M. Bamdas, *J. Gen. Chem. USSR*, **26**, 885 (1956).
(22) S. Pestka, *Arch. Biochem. Biophys.*, 136, 80 (1970).
(23) P. S. Ringrose and R. W. Lambert, *Biochem. Biophys. Acta*, **299**, 374 (1973).

Table I. UV, CD, and Inhibition Parameters of Chloramphenicol Analogues

no.	pK_a[a]	UV max, nm	ϵ max × 10^{-3}	CD max, nm	$[\theta]$ max × 10^{-3}	log k[b]	ID_{50}, M × 10^5 [c]	K_i, M × 10^6 [d]
1a				243	−0.05			
				276	−0.8			
				330	0.6			
1b	1.29			255	1.05	2.00	3	1.6
				286	−0.2			
				325	0.9			
1c	3.77	276	8.8	245	1.6		10[e]	
				299	0.15			
				335	0.55			
1d	4.76	278	9.4	246	2.2	0.48	34[e]	
				300	−0.3			
				340	0.5			
1e	4.88	279	10.2	252	2.15		39[e]	
				299	−0.05			
				333	0.65			
1f	4.82	280	9.8	247	2.5	0.71	34[e]	
				299	−0.05			
				340	0.55			
1g	4.83[f]	280	9.6	245	2.5		20[e]	
				294	−0.6			
				340	0.7			
1h	4.79[f]	279	10.0	246	2.0		10[e]	
				289	−0.5			
				335	0.75			
1i	3.83	278	14.4	247	1.65		10[e]	
				296	−0.5			
				343	0.4			
1j	2.66	278	10.3	246	1.8	1.16	27[e]	
				299	−0.2			
				340	0.4			
1k	2.86	279	10.2	252	1.25	1.71	50	27
				291	−0.45			
				335	0.65			
1l	2.43	278	9.5	250	2.05	0.14	35	1.7
				297	0.0			
				335	0.55			
1m	3.03	279	9.4	245	1.9		60[e]	22
				290	0.2			
				335	0.6			
1n		278	9.0	277	−0.1	1.84	7	1
				312	1.1			
1o	0.65	279	10.1	260	0.0	0.75	25	3
				280	−0.7			
				325	0.75			
1p	3.12[g]	261	20.8	242	0.2		10[e]	
				277	−4.3			
1q	−0.6	277	9.2	242	0.0		20	1.1
				278	−2.65			
				327	0.8			
1r	1.40	280	12.2	243	1.15		10[e]	
				280	−2.45			
				338	0.8			

[a] The values refer to the corresponding parent acids derived from substituent R in formulas 1b–r and were taken from ref 36, unless stated otherwise. [b] Generation rate constants obtained from inhibition of growth of *E. coli* cells.[17] [c] Concentration of chloramphenicol (1b) and analogues required for a 50% inhibition of *N*-acetyl-L-[^{14}C]phenylalanylpuromycin formation, unless stated otherwise. For details, see Experimental Section. [d] Inhibition constants were determined from the Dixon and slope vs. inhibitor concentration plots; see ref 24 and Experimental Section. For a typical example see Figure 3. [e] Percent inhibition at 8×10^{-4} M. [f] Reference 37. [g] Reference 38.

examine an available series of analogues, **1c–r**, as inhibitors of the puromycin reaction and, where possible, to relate the results to biological data obtained with whole cells. Compounds **1a–r** were tested by using *E. coli* 70S ribosomes, poly(U) as mRNA and *N*-AcPhe-tRNA as a peptide donor.[24] The results of these assays are sumarized in Table I, which includes, for comparison purposes, the biological data obtained by microbial kinetics in the *E. coli* whole cell system.[17] A typical inhibition plot is shown for **1b** and the methylsulfonyl analogue **1q** in Figure 2. The inhibition constants K_i were determined[24] for the most active compounds **1b**, **11**, **1o**, and **1q** from the corresponding Dixon and slope vs. inhibitor concentration plots as shown in Figure 3 for the methylsulfonyl analogue **1q**. In all cases, a competitive type of inhibition was observed.

Dibromoacetyl (**1n**) and, surprisingly, methylsulfonyl derivative **1q** were found to be slightly better inhibitors than **1b** itself. In addition, the methylsulfonamide **1q** is the first strongly active chloramphenicol analogue which does not contain a carbonyl (amide) group. However, in a whole cell assay, the inhibitory activity of compound **1q** was much lower.[25] A difference between a cell-free and whole cells assay was noted also in the case of cyanoacetyl

(24) C. Li, P. Bhuta, and J. Žemlička, *Biochemistry*, 17, 2537 (1978).

(25) M. C. Rebstock, personal communication.

derivative **11**, which is a strong inhibitor of puromycin reaction, equal to **1b**. By contrast, compound **11** exhibited only 7% of the activity of **1b** according to microbial kinetics[17] but a 60% efficiency in an antibacterial assay.[20] Inhibitory activity of the dibromoacetyl (**1n**) and trichloroacetyl (**1o**) analogues is roughly comparable in both the puromycin reaction and microbial assays.[17,26] In agreement with our data, analogue **1n** had 100% or better activity than **1b** in an *E. coli* cell-free system (endogenous mRNA).[26] Differences between in vivo and in vitro inhibition with chloramphenicol (**1b**) have been observed before[1,23] and are best explained in terms of a composite receptor site on ribosomes and its response toward changes in mRNA and aminoacyl-tRNA and type of assay. The chloroacetyl (**1k**) and azidoacetyl (**1m**) analogues each inhibited the puromycin reaction about one order of magnitude less effectively than **1b**. By contrast, microbial assays[17,20] showed that **1k** retained 60–85% of chloramphenicol activity.

Analogues **1d–g** and **1j** comprise a group of inhibitors of moderate to low activity, and the results of the inhibition of the puromycin reaction are in good agreement with other types of assays.[17,20] Surprisingly, the fluoroacetyl derivative **1j** exhibits a small inhibitory activity even though fluoroacetic acid ranks among strong acids (Table I), whereas the microbial assay[17] of **1j** indicated ca. 60% the activity of **1b**. However, the inhibitory activity trend chloroacetyl > fluoroacetyl is well explained by an empirical relationship derived from consideration of both electronic and steric effects. It is also clear from the results of the inhibition of the puromycin reaction with compounds **1b–r** that pK_a values, which related to the inductive effect of substituents in the corresponding parent acids, cannot alone account for the findings without invoking other factors. Thus, although all biologically potent analogues, **1k–o** and **1q**, are derived from strong acids, some similar analogues, such as the fluoroacetyl (**1j**) and chlorophosphonyl (**1r**) derivatives, are either borderline or exhibit essentially no activity. Similar conclusions were reached previously[17,20] on the basis of microbial assays.

Analogues **1c–f**, derived from the first four members of a homologous series of aliphatic carboxylic acids, are only poor inhibitors of the puromycin reaction. Likewise, the cyclopropyl and cyclobutyl analogues **1g** and **1h** exhibit slight activity, although compound **1g** was reported[23] to inhibit mitochondrial protein synthesis with moderate effectiveness. The lack of activity of the hydroxyacetyl analogue **1i** is somewhat surprising and may probably be attributed to the difference in mRNA. Thus, the structurally similar aminoacetyl (glycyl) analogue is a good inhibitor of cell-free poly(U,C)-mediated poly(Phe) synthesis.[7,9] On the other hand, a relatively complex and bulky acyl group is probably responsible for negligible inhibitory activity of furoyl derivative **1p**. More interesting is the chloromethylphosphonyl analogue **1r** which is structurally similar to the methylsulfonyl derivative **1q** and whose CD spectrum (vide infra) is almost superimposable with that of **1q**. However, unlike **1q**, which is a strong inhibitor, compound **1r** is inactive. It is of interest to note that phosphonyl analogues of 2′(3′)-*O*-phenylalanyladenosine, either as an isomeric mixture[27] or as pure 2′- and 3′-isomers (**7a** and **7b**), do not serve as acceptors for *N*-AcPhe-tRNA in an *E. coli* ribosomal system nor do they inhibit the puromycin reaction in the same system.[28]

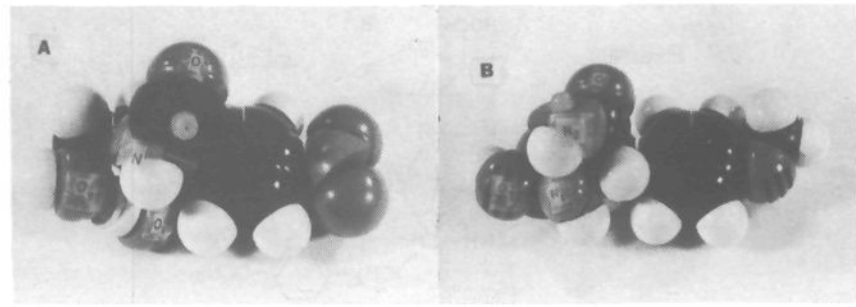

Figure 4. Space-filling models of chloramphenicol (**1b**) and a hypothetical transition state of the peptidyltransferase-catalyzed formation of peptidylpuromycin: (A) chloramphenicol (**1b**) in conformation **2**; O_1 and O_3 denote the corresponding oxygen atoms (see formula **1**) and N and O the respective atoms of the CONH function. The dichloromethyl group is omitted for clarity. (B) Transition state as shown in formula **10** (the only parts shown are those drawn in strong lines). The ribose and base portion of puromycin, the peptide chain, and tRNA are omitted for clarity. The N_1 denotes the aminoacyl amine group of puromycin, O_T represents the oxygen atom arising from the carbonyl (ester) group of peptidyl tRNA, and N_2 and O are the respective atoms of the puromycin CONH function. Note similarities in the position of the carbonyl (amide) groups and in the O_1–O_3–N triangle (A) with that of N_1–O_T–N_2 (B).

From all chloramphenicol analogues investigated to date, compound **1r** probably resembles CMP the closest, assuming a similar conformation to that found in **1b** (formula **2**). A complete lack of inhibitory activity of **1r** then argues against such a relationship.

Our results, as well as previous studies, fail to provide clear-cut evidence in favor of either possibility of chloramphenicol action indicated at the outset. It seems likely that other explanations have to be sought to account for the biological activity of **1b** and the structure–activity relationship. The initial step in this direction was undertaken in a theoretical study[29] using computational techniques. Chloramphenicol (**1b**), together with other antibiotics (lincomycin), were related to a peptide backbone in peptidyl-tRNA bound to the P site and to the corresponding transition state involving aminoacyl-tRNA and a catalytic center on the ribosome. The major drawback to this model is a lack of recognition that **1b** is essentially an A-site inhibitor.[1,2] Also, no specific role was assigned to the amide group of **1b**, although the importance of this function was acknowledged.[29] Moreover, with a single exception,[17] it was also supported by other studies.[20,30] More recent evidence for a role of the carbonyl (amide or ester) group for the A-site substrates comes from experiments with "reduced" analogues of puromycin[31] and 2′(3′)-*O*-L-phenylalanyladenosine.[32] Thus, replacement of the CO function with CH_2 led in both cases to a complete loss of acceptor and inhibitory activity.

We would, therefore, like to propose another model of chloramphenicol action which is more in line with current biochemical evidence, including the results of this study. The following hypothetical transition states, which differ in the mode of hydrogen bonding, can be postulated for the peptidyltransferase-catalyzed reaction of peptidyl-tRNA with aminoacyl-tRNA or puromycin (formulas **10** and **11**). Thus, in structure **10** the CO group becomes available for ribosomal binding, whereas in **11** it stabilizes the transition state by hydrogen bonding. In contrast to the previously proposed model,[29] this hypothesis does not consider a catalytic center on the ribosome, although it is

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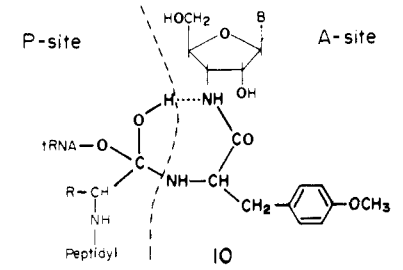

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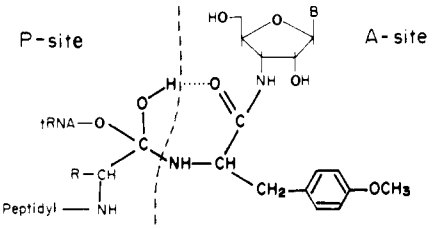

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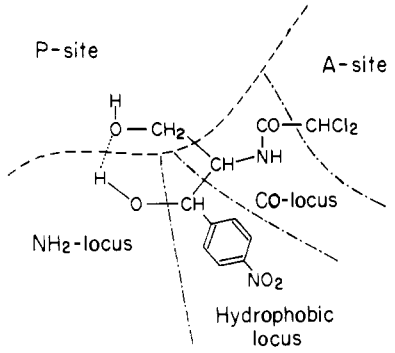
not necessarily at variance with the possibility of formation of a covalent "peptidyl ribosome" intermediate. Both transition states resemble chloramphenicol (**1b**, formula 3). Other similarities are revealed by examination of space-filling models (Figure 4). It is clear that the aromatic and amide moieties of **1b** can be related to the corresponding function in puromycin. The secondary hydroxy group (O_1) then simulates the amino function of puromycin, whereas the primary alcohol (O_3) mimics the carbonyl (ester) of peptidyl-tRNA or the corresponding hydroxy group in the transition state **10** or **11**. In this model, most of the chloramphenicol molecule, the *p*-nitrophenyl, amide, and O_1 groups, is confined to the A site, but some overlap with the P site (O_3) also occurs (formula 12). The "borderline" position of **1b** is supported



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by in vitro affinity-labeling experiments[1] with iodamphenicol and bromamphenicol which implicated both A-site (L 16) and P-site ribosomal proteins (L 2 and L 27) in the binding. The importance of the "active center", comprised of amide group, O_1, and O_3 atoms, for biological activity of **1b** was recognized earlier.[21] However, it has also been stated[9] that the chloramphenicol molecule does not correspond to aminoacyl moieties in aminoacyl-tRNA or puromycin. As already mentioned (vide infra), this view is at variance with more recent experiments.[15] In addition, the *p*-methoxyphenyl analogue of **1b**, whose aromatic moiety is equivalent to that found in puromycin, is a good inhibitor of microbial growth.[17,20] It may be argued that it is difficult to relate an L-phenylalanine moiety in Phe-tRNA or puromycin to **1b**, which has a D configuration. However, we would like to emphasize that the *direction* of the CONH sequence of amide bonds in puromycin and **1b** located in an asymmetric environment is opposite (Figure 4). Thus, with respect to the side chain atached to the aromatic moiety, **1b** can be regarded as a retro-inverso[33] analogue of the aromatic amino acid ester (amide) in Phe-tRNA (puromycin). Although many retro-inverso analogues of peptides[33] were studied and some were found to exhibit high biological activity, the concept has not yet been applied to systems containing a single CONH group. In addition, the relationship of chloramphenicol to puromycin may be the first recognized example of retro-inverso analogues found in nature.

According to the transition state **10**, carbonyl groups of puromycin and **1b** can be bound to the same locus at the A site (Figure 4). The lack of inhibitory properties[9] of "mixed" chloramphenicol and puromycin analogue **6a** is simply explained by its resemblance to peptidylpuromycins which have an understandably low affinity for the A site. By contrast, a weak inhibitory[9] activity of **5a** may reflect the capability of a highly electronegative dichloromethyl group to enhance binding affinity of the carbonyl group even in the absence of other favorable factors (aromatic moiety). Similarly, a potent inhibitory effect of **1b** and some of its analogues derived from strong acids, including compound **1q**, can be explained. In the latter case, a highly polarized S–O function can effectively replace the "activated" carbonyl group. However, a substituent with a net negative charge, such as the chloromethylphosphonyl group of analogue **1r**, has a detrimental influence probably because of an unfavorable electrostatic interaction at the active site.

This model can also account for the importance of both electronegativity and "critical" size of the acyl groups for the biological activity of **1b** and its analogues. It can be shown that the dichloromethyl group of **1b** and the ribofuranose portion of puromycin extend to approximately the same region. Thus, the alkyl moiety of an *N*-acyl group may sterically interfere with the binding of the ribofuranose portion of puromycin or aminoacyl-tRNA. In such a fashion, an overall effect of a strongly electronegative, but small, substituent, e.g., fluoromethyl in analogue **1j**, can be lower than that of a more bulky and less electronegative group (analogue **1k**).

The hypothetical model of chloramphenicol action, presented herein, provides an explanation of structure–activity and inhibitor–receptor site relationships which has not been possible by previous interpretations. It may afford a rationale for novel biologically active chloramphenicol and puromycin analogues. In addition, some close similarities in the structure and function of **1b** to other antibiotics that inhibit protein synthesis, e.g., sparsomycin and lincomycin, are certainly worthy of further exploration in light of the above model.

Experimental Section

Starting Materials. Chloramphenicol (**1b**) and the corresponding base (**1a**) were commercial products. Analogs **1c–q** were generous gifts from Drs. H. M. Crooks and M. C. Rebstock, Pharmaceutical Research Division, Warner-Lambert/Park-Davis, Ann Arbor, Mich. The purity of samples was checked by UV spectra (Table I) and TLC in a dichloromethane–methanol solvent system, 95:5 and 9:1. All samples with the exception of compound **1q** were uniform. A trace of a faster moving impurity in **1q** was removed by preparative TLC. Compound **4** was obtained through the courtesy of Dr. J. G. Moffatt, Institute of Molecular Biology, Syntex, Palo Alto, Calif.

(Chloromethyl)phosphonyl Analogue of Chloramphenicol (1r). A solution of chloramphenicol base (**1a**; 424 mg, 2 mmol), (chloromethyl)phosphonic acid (0.78 g, 6 mmol) and dicyclohexylcarbodiimide (DCC; 413 mg, 2 mmol) in 50% aqueous *tert*-butyl alcohol (20 mL) was adjusted to pH 8.5 with tri-

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ethylamine and then refluxed for 2 h. Three 2-mmol portions of DCC were added every hour until almost all starting material **1a** was converted to the product as shown by paper electrophoresis in 0.02 M Na_2HPO_4, pH 7. The precipitated dicyclohexylurea was filtered off and the filtrate was evaporated in vacuo. The residue was dissolved in 50% methanol and applied on a DEAE-cellulose column (HCO_3^- form, 3.5 × 70 cm), which was eluted with a linear gradient of triethylammonium bicarbonate in 50% methanol (0.05 M, 2 L in the reservoir, 2 L of 50% methanol in the mixing vessel, flow rate 1.3 mL/min, 20-mL fractions were taken). The chromatography was monitored at 254 nm by a LKB Uvicord II (Uppsala, Sweden). The major peak was evaporated, the residue coevaporated several times with methanol, and the product **1r** was converted to a sodium salt by passage of its aqueous solution through Dowex 50 resin in Na^+ form. The eluate was lyophilized, the residue was dissolved in methanol (3 mL), and compound **1r** was precipitated by the addition of ether (100 mL) and dried in vacuo, yield 430 mg (82%). Product **1r** was uniform on paper electrophoresis (phosphate, pH 7, mobility −0.7 of chloramphenicol base **1a**) and chromatography (ethanol–1 M ammonium acetate, 5:2; R_f 0.73). For UV and CD spectra, see Table I. Anal. Calcd. N/P 2, found N/P 1.8; C, H, N (−0.67), P.

Separation of 2′- and 3′-*O*-(*R*,*S*)-[1-[(Benzyloxycarbonyl)amino]-2-phenylethane-1-phosphonyladenosine (7c and 7d). The isomeric mixture of **7c** and **7d** prepared as described[27] (triethylammonium salt; 185 mg, 0.27 mmol) was dissolved in water and the pH of the solution was adjusted to 8.5 with triethylammonium bicarbonate before it was applied on the top of the Dowex 1-X 2 column (formate; 2 × 58 cm).[34] Elution with water and 50% methanol (200 mL) was followed with a linear gradient of sodium formate (pH 5.0; 2 L of 0.05 M in the mixing vessel and 2 L of 0.08 M in the reservoir; flow-rate 2.5 mL/min; 13-mL fractions were taken; for monitoring, see the preceding experiment). The 2′-isomer (**7c**) was eluted first at 0.062–0.065 M and the 3′-isomer (**7d**) at 0.066–0.07 M. The appropriate fractions were pooled and each was desalted with Dowex 50 (pyridinium form). The resin was washed with 50% pyridine and the eluates were evaporated to give pyridinium salts of **7c** (85 mg, 47.5%) and **7d** (65 mg, 36%). Both were converted to sodium salts using NaI in acetone: **7c** (55 mg, 34%) and **7d** (60 mg, 36%). Mobility in phosphate, pH 7, relative to adenosine 5′-phosphate: 0.45 (**7c**) and 0.33 (**7d**); UV max (water) 260 nm (ε 11 800, **7c**; 10 700, **7d**); NMR (100 MHz, CD_3SOCD_3), **7c**, δ 7.71 (s, H_8), 7.45 (s, H_2), 6.62 (m, C_6H_5), 5.68 (d, $H_{1'}$, $J_{1',2'} \approx 5$ Hz); **7d**, δ 7.65 (s, H_8), 7.45 (s, H_2), 6.62 (br s, C_6H_5), 5.43 (d, $H_{1'}$ $J_{1',2'} \approx 5$ Hz).

2′- and 3′-(*R*,*S*)-(1-Amino-2-phenylethane)phosphonyladenosine (7a and 7b). Compounds **7c** and **7d** (20 mg of each, 35 μmol) were deprotected by hydrogenolysis as described for the corresponding isomeric mixture.[27] The products were purified by TLC on Avicel microcrystalline cellulose layers in 2-propanol/NH_4OH/water (7:1:2), the appropriate UV-absorbing bands were eluted with the same solvent, the eluates were evaporated, and the residues were lyophilized from water; yield 10 mg each of **7a** and **7b** (65%); mobility in phosphate, pH 7, relative to adenosine 5′-phosphate 0.56 (**7a**) and 0.47 (**7b**); R_f 0.50 (**7a**) and 0.24 (**7b**) relative to adenosine 5′-phosphate in saturated aqueous $(NH_4)_2SO_4$/NH_4OH/2-propanol (79:19:2); UV max (water) 260 nm (ε 9800, **7a**; 11 500, **7b**).

CD Measurements. The general technique employed was described before.[35] The CD spectra were scanned in a 1-cm cell in aqueous solutions whose pH was adjusted to 7 with NaOH between 230 and 400 nm at a concentration of 0.5–1.5 × 10^{-4} M. The results were digitized by hand and plotted as molar ellipticities [θ] against the wavelength (Figure 1).

Ribosomal Peptidyltransferase Assay. The ability of compounds **1a–r** and **4** to inhibit the puromycin reaction was measured as described before[24] using *E. coli* MRE-600 70S ribosomes and *N*-Ac[^{14}C]Phe-tRNA. Compounds **7a** and **7b** were neither acceptors of the *N*-AcPhe residue nor inhibitors in this system.[28] A typical reaction mixture contained, in 0.1 mL, 0.05 M Tris-HCl (pH 7.4), 0.1 M NH_4Cl, 0.01 M $MgCl_2$, 4.0 A_{260} units of NH_4Cl-washed ribosomes, 10 μg of poly(U), and 0.20 A_{260} unit (2000 cpm) of *N*-Ac[^{14}C]Phe-tRNA, puromycin, and inhibitors **1a–r** and **4** at the desired concentrations. The reactions were incubated at 37 °C for 30 min, whereupon they were stopped by the addition of 0.1 M $Be(NO_3)_2$ in 0.3 M sodium acetate buffer (pH 5.5), saturated with $MgSO_4$, and the products were extracted with ethyl acetate (1.5 mL). One milliliter of the ethyl acetate layer was transferred into a scintillation vial and the radioactivity was determined as specified before.[24] Dixon plots used for calculating the K_i values were constructed using a linear-regression analysis.

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