Aminoacyl Analogs of Chloramphenicol: Examination of the Kinetics of Inhibition of Peptide Bond Formation

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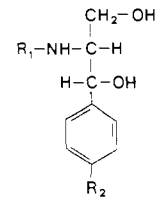
Two aminoacyl analogs and one peptidyl analog of chloramphenicol (1, Cl_2CHCO-CA) were prepared. These are **2** (L-Phe-CA), **3** (Gly-CA), and **4** (L-Phe-Gly-CA). The kinetics of inhibition of peptide bond formation by these analogs were examined in a cell-free system which was derived from *E. coli* and used previously for the study of 1 (Drainas; et al. *Eur. J. Biochem.* **1987**, *164*, 53-58). In the absence of inhibitor, the reaction follows first-order kinetics for the entire course of the reaction. In the presence of the analog the reaction gives biphasic log-time plots. The kinetic information pertaining to the *initial slope* of the plot is analyzed (initial-slope analysis). This information differentiates the analogs from the parent compound 1. The parent compound 1 gives complex inhibition kinetics; increasing the concentration of 1 changes the inhibition from competitive to mixed noncompetitive (Drainas; et al. *Eur. J. Biochem.* **1987**, *164*, 53-58). In contrast, the analogs give competitive kinetics even at high concentrations of the inhibitor. The following K_i values have been determined: 18.0 μM for **2**, 5.5 μM for **3**, 1.5 μM for **4**. If we were to assume that compounds **2**, **3**, and **4** behave as classical competitive inhibitors, we could say that **4** is 12 times more potent than **3** and 4 times more potent than **2**. On this assumption we could also compare **1** with **4** and see that **4** is 2 times weaker than **1**. It is suggested that as compared with **1**, the two aminoacyl analogs and the dipeptidyl analog have increased structural similarity to the 3′-terminus of aminoacyl-tRNA or of peptidyl-tRNA and that this similarity results in a more pronounced competitive inhibition.

Introduction

Aminoacyl analogs of chloramphenicol in which the dichloroacetyl moiety is replaced by aminoacyl residues have been used[1,2] with the aim of examining the following hypothesis: (1) Chloramphenicol (**1**) inhibits protein synthesis by acting as a conformational analog of the peptidyl adenylyl terminus of peptydyl-tRNA and might compete on the ribosome for sites normally occupied by peptidyl-tRNA or aminoacyl-tRNA.[1] In this hypothesis, the exact structural relationship between **1** and the 3′-end of the aminoacyl-tRNA or of peptidyl-tRNA was not defined. One of several possibilities suggested by this hypothesis was the analogy between the aminoacyl residue of these analogs and the *p*-methoxy-Phe residue of puromycin.[3] Harris and Symons[4] have proposed another possibility which, in agreement with our hypothesis (1), presents **1** as an analog of the peptidyl–adenylyl terminus of peptidyl-tRNA; more precisely an analog of the carboxyl terminus of the nascent peptide as previously proposed by Das et al.[5] and by Hahn.[6] In both models the aminoacyl analogs of **1** "have increased structural similarity to the 3′-terminus of aminoacyl-tRNA and of a nascent peptide", as Harris and Symons[4] note. McFarlan and Vince[7] have introduced compound **4** and reported that it is an inhibitor of peptide bond formation at least as strong as **1**. There is still great interest in the elucidation of the mechanism of action of **1** in inhibiting protein synthesis.[8-11] The availability of peptidyl analogs of **1**, along with the aminoacyl analogs, could provide useful information. However, comparisons in potencies of **1** and its analogs cannot be made unless we assume knowledge of kinetic models for which well-defined equilibrium and/or kinetic constants can be determined. We have already presented evidence[8] that **1**, thiamphenicol (**1b**), and tevenel (**1c**) do not behave as classical competitive inhibitors when the puromycin reaction is used as a model of ribosomal peptide bond formation; additional parameters, besides the K_i, are needed for full characterization of potency. Using the same experimental system we have now examined the inhibition caused by **4** as well as by **2** and **3** which are close analogs of **4** (Figure 1). The results are reported in the present paper.



R_1	R_2	COMPOUND
Cl_2CHCO-	$-NO_2$	1
H-	$-NO_2$	1a
Cl_2CHCO-	$-SO_2CH_3$	1b
Cl_2CHCO-	$-SO_2NH_2$	1c
CH_3CO-	$-NO_2$	1d
NH_2CHCO- CH_2 C_6H_5	$-NO_2$	2
NH_2CH_2CO-	$-NO_2$	3
$CH_3CONHCH_2CO-$	$-NO_2$	3c
$NH_2CHCONHCH_2CO-$ CH_2 C_6H_5	$-NO_2$	4

Figure 1. Structures of chloramphenicol and its derivatives.

Experimental Section

General. Chloramphenicol free base (D-(−)-*threo*-1-(*p*-nitrophenyl)-2-amino-1,3-propanediol) (**1a**) was a product of Sigma. Trityl amino acids[12] and 1-hydroxybenzotriazole ester of *N*-trityl-L-phenylalanine (**5**[13]) were synthesized according to known methods. *N*-Hydroxysuccimide ester of tritylglycin (**6**, mp = 146 °C) was prepared in 67% yield from tritylglycin by the method of Anderson et al.[14]

The purity of samples was checked by TLC in chloroform-methanol (9:1) (system A) or in a butanol–acetic acid–water (2:3:5) (system B). Melting points were obtained with a digital melting point apparatus (Electrothermal) and are not corrected; infrared spectra were obtained with a Perkin-Elmer infrared

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spectrophotometer, Model 257. Mass spectra were obtained with a mass spectrometer–data system MAT 312/55220 (Varian-Finnigan-MAT Co.) equipped with FAB apparatus (Ion Tech Ltd.). All IR and mass spectra were consistent with the assigned structures. Optical rotations were determined with a Carl Zeiss precision polarimeter.

(A) Preparation of *N*-tritylaminoacyl and Dipeptidyl Analogs of Chloramphenicol (2a, 3a, and 3b). (1) D-(−)-***threo*-1-(*p*-Nitrophenyl)-2-(*N*-tritylglycylamido)-1,3-propanediol (3a).** A solution of **1a** (2.31 g, 11.0 mmol) in dry dimethylformamide (7 mL) and triethylamine (1 mL) was cooled at 0 °C. **6** (3.15 g, 10 mmol) was added to the above solution with stirring. The mixture was incubated for 20 min at 0 °C and for 3 h at room temperature with continuous stirring. The resulting solution was partitioned between 10 mL of a saturated solution of sodium chloride (SSSC) and 70 mL of ethyl acetate. The ethyl acetate layer was extracted twice with 10 mL of 5% citric acid, once with 10 mL of 10% sodium carbonate, and with 10 mL of SSSC. The ethyl acetate layer was dried over $MgSO_4$ and evaporated *in vacuo*. The resulting light-yellow powder was recrystallized from ethyl acetate and yielded 3.74 g (76%) of white crystals of **3a**: mp 212–214 °C; TLC (system A) R_f 0.74; $[\alpha]^{25}_D$ = −19.1 (*c* = 1, DMF).

(2) D-(−)-*threo*-1-(*p*-Nitrophenyl)-2-(*N*-trityl-L-phenylalanylamido)-1,3-propanediol (2a). A solution of **1a** (2.31 g, 11.0 mmol) in dry dimethylformamide (8 mL) and triethylamine (1 mL) was cooled at 0 °C. **5** (5.24 g, 10.0 mmol) was added to the above solution with stirring. The mixture was incubated for 20 min at 0 °C and for 4 h at room temperature with stirring. The resulting solution was partitioned between 15 mL of SSSC and 70 mL of ethyl acetate. The ethyl acetate layer was dried over $MgSO_4$ and evaporated *in vacuo*. The resulting light-yellow powder was recrystallized from ethyl acetate and yielded 5.05 g (84%) of white crystals **2a**: mp 143–144 °C; TLC (system A) R_f 0.71; [a]$^{25}_D$ = −48.1 (*c* = 1.1, $CHCl_3$).

(3) D-*threo*-1-(*p*-Nitrophenyl)-2-(*N*-trityl-L-phenylalanylglycylamido)-1,3-propanediol (4a). A solution of **3b** (4.85 g, 11.0 mmol) in dry dimethylformamide (8 mL) and triethylamine (2 mL) was cooled at 0 °C. **5** (5.24 g, 10.0 mmol) was added to the above solution with stirring. The mixture was kept with stirring at 0 °C for 20 min and then overnight at room temperature. The resulting solution was processed up as in the case of **3a** and yielded 5.34 g (81%) of white foam of **4a**: TLC (system A) R_f 0.80.

(B) Preparation of the Tosylates of Aminoacyl and Dipeptidyl Analogs of Choramphenicol (2b, 3b, and 4b). **2a**, **3a**, or **4a** (10 mmol) was dissolved in 15 mL of a solution of 15.0 mmol of toluene-4-sulfonic acid monohydrate in isopropyl alcohol. The mixture was heated at 60 °C for 20 min, and the tosylate was crystallized by standing at room temperature overnight. The white crystals were filtered under vacuum, washed with small aliquots of ether, and recrystallized from isopropyl alcohol. The yields were 3.66 g (83%) of **3b** [mp 220–222 °C; TLC (system B) R_f = 0.28]; 4.25 g (80%) of **2b** [mp 164–165 °C; TLC (system B) R_f = 0.55]; 4.18 g (71%) of **4b** [mp 139–140 °C; TLC (system B) R_f = 0.37].

(C) Preparation of Aminoacyl and Dipeptidyl Analogs of Chloramphenicol **(2–4).** (1) D-(−)-***threo*-1-(*p*-Nitrophenyl)-2-(L-phenylalanylamido)-1,3-propanediol (2) and** D-(−)-***threo*-1-(*p*-nitrophenyl)-2-(L-phenylalanylglycylamido)-1,3-propanediol (4)** were obtained as free bases from their tosylates. Then 5 mmol of **2** or **4** was partitioned between 60 mL of ethyl acetate and 10 mL of 10% sodium carbonate. The ethyl acetate layer was extracted three times with 10 mL of SSSC dried over $MgSO_4$ and eveporated under vacuum. The yields were (a) 850 mg (47.26%) of **2** [IR (KBr) 3460, 3320, 1640, 1510, 1340, 1142, 1070, 1010, 848, 755 cm^{-1}; MS (FAB) *m/z* 360 (22) (M + H); UV_{max} (2% CH_3COOH) = 277 nm (ϵ = 8567); $[\alpha]^{25}_D$ = −57.2 (*c* = 1 DMF)]; (b) 720 mg (34.6%) of **4** [IR (KBr) 3300–3060, 1650, 1600, 1520, 1340, 1070, 850, 755 cm^{-1}; MS (FAB) *m/z* 417 (9) (M + H); UV_{max} (2% CH_3COOH) = 279 nm (ϵ = 8394); $[\alpha]^{25}_D$ = −27.6 (*c* = 1 DMF)]. Elemental analysis gave the following data: calculated for $C_{20}H_{24}N_4O_6$ (MW 416.42) C, 57.68; H, 5.81; N, 13.46. Found: C, 57.61; H, 5.72; N, 13.58.

(2) The free base D-(−)-***threo*-1-(*p*-nitrophenyl)-2-(glycylamido)-1,3-propanediol (3)** was obtained from **3a** as follows: 2.69 g of **3a** was suspended in 3 mL of glacial acetic acid, and the mixture was heated at 60 °C for 20 min. The resulting solution was cooled at room temperature, and cold ether was added. The product precipitated immediately, and the precipitate was dissolved in a small aliquot of ethyl acetate and reprecipitated with ether. This procedure was repeated until the triphenylcarbinol was removed completely [IR 3280–3100, 1670, 1570, 1515, 1347, 1060, 1040, 855, 755, cm^{-1}; MS (FAB) *m/z* (%) 270 (13) (M + H); UV_{max} (2% CH_3COOH) = 278 nm (ϵ = 7416); $[\alpha]^{25}_D$ = −30.1 (*c* = 1, DMF)].

(D) Peptide Bond Formation in a Cell-Free System. The system was derived from *E. coli*. The [Ac^{3}H-Phe-tRNA 70S ribosome poly(U)] complex (complex C) was prepared and purified by adsorption on cellulose nitrate filter disks as reported earlier.[15] The amount of Ac[^{3}H]Phe-tRNA bound to complex C contained in a half disk was 10 pmol (95 300 cpm). The puromycin reaction was carried out either *without preincubation* (the disk-absorbed complex C reacted with a mixture of puromycin and inhibitor) or *after preincubation* (10 min, 25 °C) of the disk absorbed complex C with the inhibitor.[15] The first-order analysis of the puromycin reaction in the absence of inhibitor has been described elsewhere.[15,16] The entire course of the reaction in the absence of inhibitor obeys pseudo-first-order kinetics. In the presence of inhibitor of first-order time plots were biphasic. The initial slope (*k*), i.e. the slope of the line going through the origin, is taken as the apparent first-order rate constant (*initial slope analysis*). The initial slopes of the first-order time plots at the various concentrations of puromycin [S] obey the relationship $k = k_{max}[S]/(K_S' + [S])$ where K_S' is the apparent K_s in the presence of the inhibitor (see also refs 8 and 15).

Results

In the absence of inhibitor the reaction between complex C (C) and excess puromycin (S) is given by eq 1 where P

$$C + S \underset{}{\overset{K_s}{\rightleftharpoons}} CS \xrightarrow{k_3} P + C' \qquad (1)$$

is the product (Ac-Phe-puromycin) and C′ is a form of complex C that cannot be reconverted to C. The puromycin reaction can be analyzed as a pseudo-first-order reaction[16] giving linear log–time plots. Such a plot at 100 μM puromycin is given in Figure 2 (uppermost line). The progress curves after preincubation of complex C with **4**, **3**, and **2** are also shown in Figure 2. There was no substantial difference in the progress curves when the puromycin reaction was carried out without preincubating complex C with the analogs.

In order to construct the double reciprocal plot ($1/k$ vs $1/S$) we determined the initial slopes (*k*) of the first-order time plots. Figure 3 shows double reciprocal plots with increasing concentration of **4**. The lowest line represents the data obtained in the absence of inhibitor (control). The heavy line represents the results in the presence of **1** at 10 μM when the puromycin reaction was carried out after preincubation of complex C with inhibitor. Under these conditions at 10 μM **1** is a stronger inhibitor than **4**, whose potency does not change after preincubation. There is indeed a striking difference between **1** and **4**. Whereas analog **4** shows competitive kinetics, **1** does not. The initial slopes analysis of the first-order time plots and the construction the double reciprocal plots offers a kinetic discrimination between the parent compound **1** and analog **4**. The slopes of the lines of Figure 4 were replotted against the concentration of the analog, and the results are shown in the inset of Figure 4. This slope replot is indicative of simple competitive inhibition and leads to the graphical determination of K_i = 1.5 μM from the negative intercept of the line with the *I*-axis. Further evidence for the simple competitive kinetics comes from the Dixon plot which is

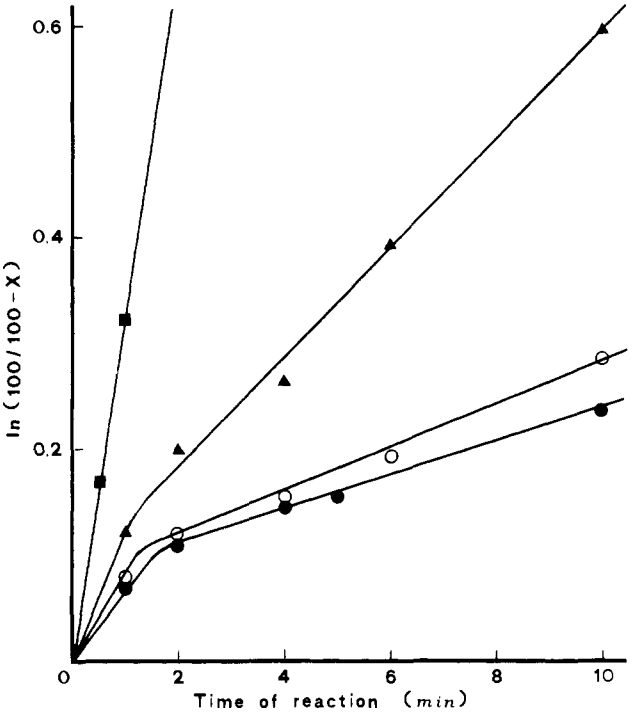

Figure 2. First-order time plots for AcPhe-puromycin formation in the absence or presence of 4. Complex C was preincubated for 10 min with 4, and then puromycin at a final concentration of 100 μM was added. (●) 10 μM of 4; (○) 10 μM of 3; (▲) 10 μM of 2; (■) without inhibitor (control).

shown in Figure 4. When the slopes of the lines of Figure 4 are plotted against 1/puromycin (S) the replot (Figure 4, insert) is a straight line passing through the origin. This is further evidence for the simple competitive inhibition and differentiates competitive from mixed noncompetitive inhibition which would give a similar Dixon plot but a different slope replot.[17] The same kinetic examination was carried out for the two other analogs, 2 and 3. The results were completely analogous. They showed simple competitive inhibition with the K_i values given in Table I. In this table we present for comparison also the K_i values obtained in another study[8] for 1, 1b, and 1c (structures in Figure 1).

Discussion

We have followed chemical standard methods of synthesis in order to prepare the aminoacyl and the peptidyl analogs. Analog 4 has appeared in the literature,[7] but no chemical data were reported. Rebstock and Stratton[18] have synthesized several α-amino acid amides of DL-*threo*-1-(*p*-nitrophenyl)-2-amino-1,3-propanediol including glycine and phenylalanine amides.

The results of this investigation, combined with results obtained previously,[2,8] show that the inhibition of the puromycin reaction by two sets of chloramphenicol analogs (Table I) can be differentiated kinetically.

Group A comprises 1, 1b, and 1c which, as shown previously,[8] inhibit the puromycin reaction via complex kinetics: the kinetics of inhibition are initially competitive but, with increasing concentrations of the inhibitor, mixed noncompetitive inhibition is observed.

Group B comprises the three analogs 2, 3, and 4. In the same experimental system and by the same method of analysis (initial-slope analysis) the inhibitors of this group exhibit simpler kinetics showing only competitive inhibition even at high concentrations of the inhibitor. For

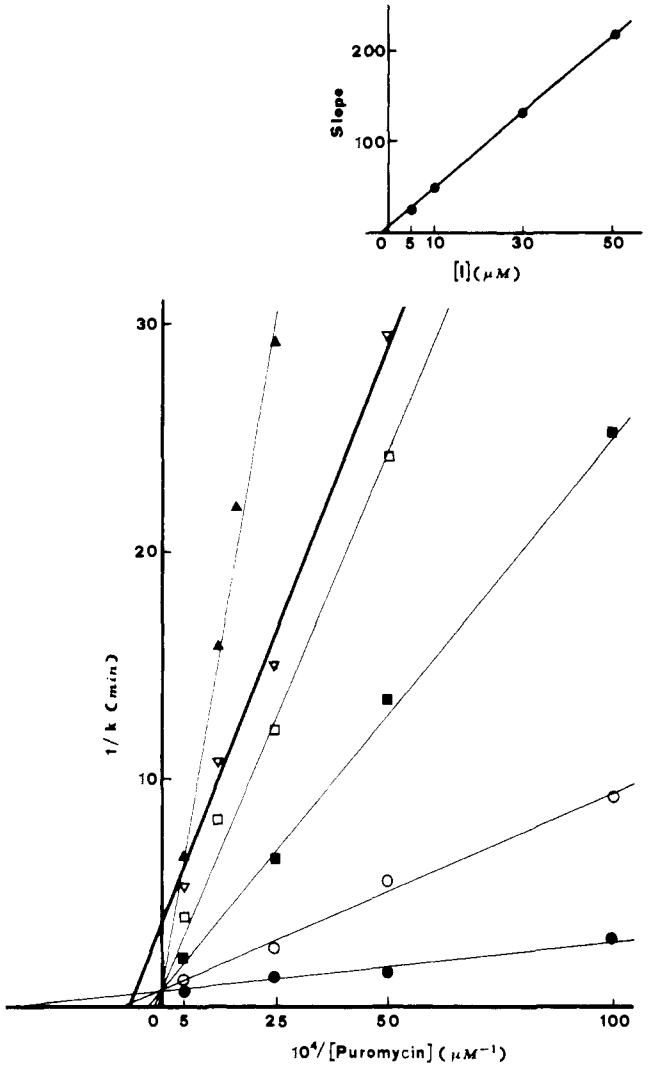

Figure 3. Double-reciprocal plot ($1/k$ versus 1/[puromycin]) for the puromycin reaction after preincubation of complex C with inhibitor for 10 min. The puromycin reaction was then carried out at each one of the indicated concentrations in the presence or in the absence of inhibitor: (●) without inhibitor; with 4 at (○) 1 μM, (■) 5 μM, (□) 10 μM, (▲) 30 μM. Heavy line (▽) 10 μM of 1. Inset: Replot of the slopes of the double-reciprocal lines versus inhibitor (I) concentration.

Table I. Equilibrium Constants Derived from Primary and Secondary Kinetic Plots

compd	K_i (μM)	compd	K_i (μM)	compd	K_i (μM)
1	0.7[a]	1c	1.7[a]	3	5.5
1b	0.4[a]	2	18.0	4	1.5

[a] Data from ref 8.

detailed schematics regarding structural similarities between analogs 2, 3, 4, and aminoacyl-tRNA or the ribosomal peptidyltransferase, the reader could consult formulas 10, 11, and 12 of ref 19 as well as Figure 1 of ref 9 and Figure 2 of ref 4.

The difference in the kinetic behavior, between aminoacyl and peptidyl analogs 2, 3, and 4 on the one hand and the parent compound 1 on the other, might suggest that the analogs mimic the aminoacyl-tRNA terminus in a more functional manner because they have an increased structural similarity to the 3′-terminus of aminoacyl-tRNA or of peptidyl-tRNA. The result of this increased similarity appears to be the simple competitive kinetics (mutually exclusive mode of binding). The competitive character of the analogs has been increased, and the mixed noncom-

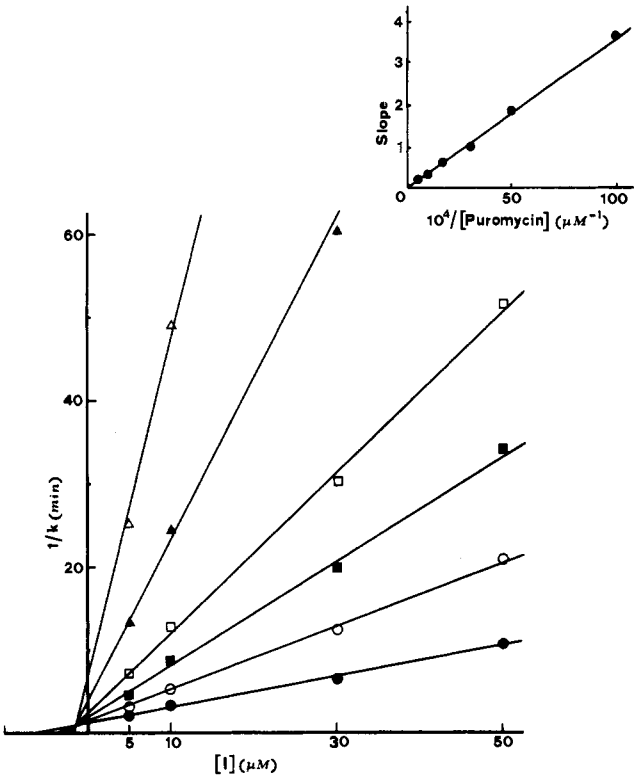

Figure 4. Dixon plot for the puromycin reaction after preincubation with analog **4** as the inhibitor (I). The concentrations of puromycin were (●) 2 mM, (○) 1 mM, (■) 0.6 mM, (□) 0.4 mM, (▲) 0.2 mM, (△) 0.1 mM. Inset: Replot of the slopes of the Dixon lines versus 1/[puromycin].

petitive inhibition phase, which is characteristic of **1**,[8] cannot be observed even at high concentrations of the analogs.

According to the present kinetic analysis we might assign potencies solely on the basis of the K_i of the competitive phase. Thus, compound **4** appears to be **2** times weaker than **1** (Table I) whereas according to McFarlan and Vince[7] **1** and **4** are equivalent in potency. However, great caution should be exercised before finalizing this comparison, as it is known that some additional constants should be used for characterizing fully the potency of **1**.[8] If, for instance, we suppose that the kinetic model comprises eq 1 and the simple equilibrium C + I ⇌ CI, then the K_i of 1.5 μM should be the only parameter that we should use. To our knowledge there are no other proposals regarding the kinetic model that should be applied in the case of **2**, **3**, and **4**. With the data we have at present we could say that **4** is 12 times more potent than **2** and 4 times more potent than **3**. It is of interest that replacement of the two chlorine atoms in **1** by one hydrogen and one amino group gives a comparatively strong inhibitor, at least stronger than compound **2**. If the two chlorines are replaced by two hydrogens (compound **1d**, Figure 1), the activity is very low.[2] It is also of interest that the "amide bond" characterizing many inhibitors of peptidyltransferase[9,20] is retained in the three analogs used in the present study. However, the acyl group must be appropriately substituted at the α-carbon. If all the substituents are hydrogens (compound **1d**) the activity is very low;[2] very low is also the activity[2] if the free amino group of **3** is acetylated (compound **3c**, Figure 1).

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