PHENYLBORIC ACIDS – A NEW GROUP OF PEPTIDYL TRANSFERASE INHIBITORS

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1. Introduction

Inhibitors of peptidyl transferase studied so far have been mainly either antibiotics or structural analogues of part of the natural substrate. In this paper we present evidence that a different group of compounds, namely phenylboric acid and its derivatives, inhibits peptidyl transferase rather strongly.

Phenylboric acid has been hitherto known as a specific inhibitor of serine proteases [1] which acts as a transition-state analogue by forming a tetrahedral adduct with the serine –OH group and the histidine residue in the catalytic centre of these proteases [2]. Phenylboric acid also forms a complex with cis-diol groups, i.e., the 2′,3′-*cis*-diol group of ribose at the 3′-terminus of RNA [3].

In this paper we describe the effect of phenylboric acid on the catalytic and binding properties of peptidyl transferase of *Escherichia coli* ribosomes.

2. Materials and methods

2.1. *Materials*

Puromycin dihydrochloride was obtained from Nutritional Biochemicals, USA. Phenylboric acid, *o*-nitrophenylboric acid, *m*-nitrophenylboric acid and *m*-aminophenylboric acid were gifts from Dr. A. Holý and Dr. I. Rosenberg of the Institute of Organic Chemistry and Biochemistry, Prague, and were prepared as in [4]. The 2′ (3′)-*O*-(*N*-formylmethionyl)-adenosine-5′-phosphate (pA-fMet) was a gift from Dr. A. A. Krayevsky, Institute of Molecular Biology, Moscow.

L-[4,5-^{3}H]Leucine (55 Ci/mmol), L-[2,4,6-^{3}H]-phenylalanine (73 Ci/mmol) and L-phenyl-[2,3-^{3}H]-alanine (16 Ci/mmol) were the products of the Radiochemical Centre, Amersham.

2.2. *Preparation of ribosomes*

Ribosomes from *E. coli* were prepared by washing with 0.5 M NH_4Cl as described elsewhere [5].

2.3. *Preparation of substrates*

Preparation of ac-[^{3}H]Phe-tRNA and CACCA-ac-[^{3}H]Leu were described earlier [6]. CACCA-[^{3}H]Phe was prepared according to Pestka [7].

2.4. *Transfer assays*

Transfer assay with CACCA-ac-[^{3}H]Leu and puromycin was carried out under the conditions of the fragment reaction described by Monro et al. [6]. The acylaminoacyl-puromycin formed was extracted according to Miskin et al. [8].

Poly(U)-directed transfer of ac-Phe-residue from ac-[^{3}H]Phe-tRNA to puromycin was measured according to [5].

The transfer reaction with pA-fMet and CACCA-[^{3}H]Phe was assayed as in [9].

2.5. *Binding of acceptor and donor substrates*

The binding of the acceptor substrate, CACCA-[^{3}H]Phe, to the ribosomes was determined in the presence of 20% ethanol according to Pestka [7].

The binding of the donor substrate, CACCA-ac-[^{3}H]Leu, to the ribosomes was examined by the method of Celma et al. [10].

3. Results

3.1. *Inhibition of peptidyl transferase activity by phenylboric acid and its derivatives*

Phenylboric acid and substituted phenylboric acids inhibited the capacity of the peptidyl transferase to form a new peptide bond assayed in three systems of varying complexity: (i) the transfer of the ac-Phe-

Table 1
The effect of phenylboric and *m*-nitrophenylboric acid on transfer reactions with various substrates

Transfer system		PBA (mM)	*m*-NPBA (mM)	Acylaminoacyl transfer	
Donor substrate	Acceptor substrate			(cpm)	(%)
ac-[^{3}H]Phe-tRNA	Puromycin	–	–	2845	100
		5	–	2361	83
		10	–	1993	70
		–	3	1903	67
		–	5	653	23
		–	10	175	6
CACCA-ac-[^{3}H]Leu	Puromycin	–	–	2450	100
		3	–	1078	44
		6	–	602	25
		10	–	198	8
		–	1.5	1122	46
		–	3	128	5
pA-fMet	CACCA-[^{3}H]Phe	–	–	2015	100
		5	–	1192	59
		10	–	743	37
		–	2.5	605	30
		–	5	263	13

The transfer reaction was examined with: (a) ribosomes (50 pmol), ac-[^{3}H]Phe-tRNA (2 pmol) and puromycin (50 nmol); (b) ribosomes (45 pmol), ac-[^{3}H]Leu-pentanucleotide (0.28 pmol) and puromycin (15 nmol); (c) ribosomes (110 pmol), pA-fMet (200 nmol) and CACCA-[^{3}H]Phe (2 pmol). PBA: phenylboric acid; *m*-NPBA: *m*-nitrophenylboric acid

residue from acPhe-tRNA to puromycin; (ii) the transfer of the ac-Leu-residue from CACCA-ac-Leu to puromycin in the fragment reaction and (iii) the transfer of the fMet-residue from pA-fMet to the terminal acceptor fragment CACCA-Phe. It is evident from the data in table 1 that all three systems are inhibited by phenylboric acid and *m*-nitrophenylboric acid and that *m*-nitrophenylboric acid is a more potent inhibitor than phenylboric acid.

Figure 1 shows the effects of different concentrations of the inhibitors on the peptidyl transferase activity assayed by the fragment reaction. As inhibitors were tested phenylboric acid and its derivatives, *o*-nitrophenylboric acid, *m*-nitrophenylboric acid, *m*-aminophenylboric acid and boric acid. *m*-Nitrophenylboric acid was the most effective inhibitor

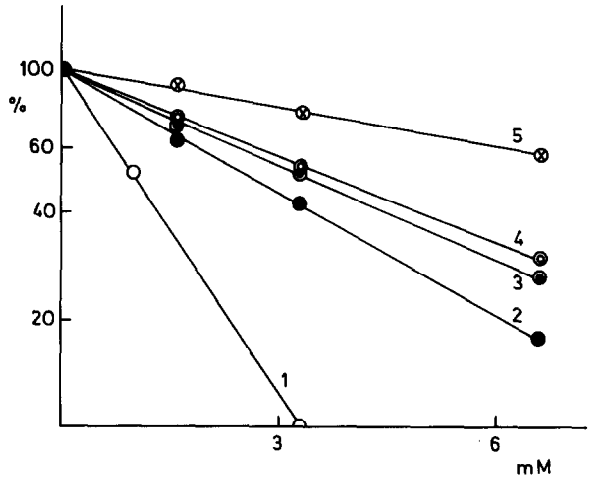

Fig.1. Inhibition of ribosomal peptidyl transferase activity by phenylboric acid, *m*-aminophenylboric acid, *m*-nitrophenylboric acid, *o*-nitrophenylboric acid and boric acid. Ribosomes (45 pmol) were examined in the fragment reaction with ac-[^{3}H]Leu-pentanucleotide (0.35 pmol) as donor substrate and puromycin (15 nmol) as the acceptor substrate. (%) ac-[^{3}H]leucyl-puromycin formation (100% transfer was 3320 cpm corresponding to 0.14 fmol acetylleucyl-puromycin min^{-1} pmol ribosomes^{-1}); (mM) concentration of inhibitors; (1) *m*-nitrophenylboric acid, (2) phenylboric acid, (3) *m*-aminophenylboric acid, (4) *o*-nitrophenylboric acid, (5) boric acid.

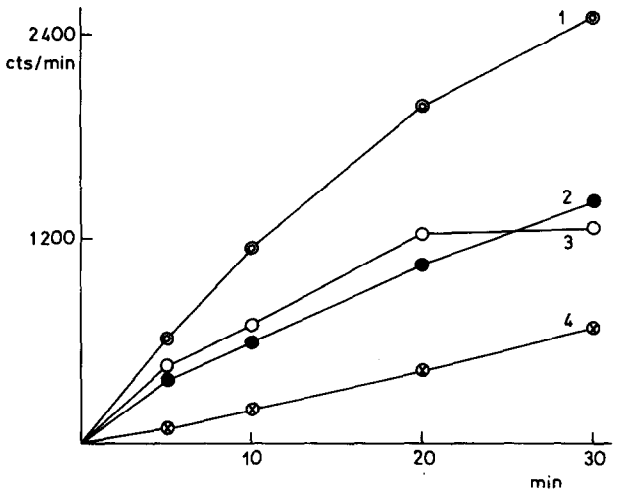

Fig.2. Time course of inhibition of peptidyl transferase activity by *m*-aminophenylboric acid, *o*-nitrophenylboric acid and *m*-nitrophenylboric acid. The effect of inhibitors (2.5 mM) was examined in the fragment reaction with ribosomes (45 pmol), ac-[^{3}H]Leu-pentanucleotide (0.28 pmol) and puromycin (15 nmol). (1) control, (2) *m*-aminophenylboric acid, (3) *o*-nitrophenylboric acid, (4) *m*-nitrophenylboric acid.

reaching 50% inhibition at 1 mM, phenylboric acid, *o*-nitrophenylboric acid and *m*-aminophenylboric acid inhibited the fragment reaction to 50% at about a 3 mM concentration. The least inhibitory effect was observed for boric acid that did not reach 50% inhibition even at the 7 mM concentration. The effect of substituted phenylboric acids on the time course of the fragment reaction is illustrated in fig.2. Also here *m*-nitrophenylboric acid showed the highest inhibitory effect.

3.2. *Competition of* m-*nitrophenylboric acid with substrates of the peptidyl transferase*

The Lineweaver–Burk plot of inhibition of the fragment reaction by *m*-nitrophenylboric acid at different concentrations of puromycin indicates that the inhibitor competes with puromycin for the binding site (fig.3). K_i for *m*-nitrophenylboric acid is 4.6×10^{-4}.

On the other hand, inhibition of the peptidyl transferase activity by *m*-nitrophenylboric acid is non-competitive with respect to the donor substrate, as shown by Lineweaver–Burk plot in fig.4.

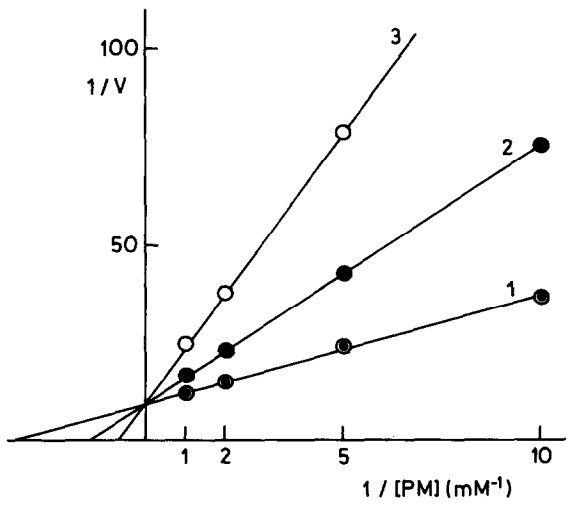

Fig.3. Inhibition of the peptidyl transferase by *m*-nitrophenylboric acid at different concentrations of puromycin. The peptidyl transferase activity was determined in the fragment reaction with 55 pmol ribosomes, 0.33 pmol ac-[^{3}H]-Leu-pentanucleotide and 1, 0.5, 0.2 and 0.1 mM puromycin. v is expressed as fmol acetylleucyl-puromycin formed min^{-1} pmol ribosomes^{-1}. The concentrations of *m*-nitrophenylboric acid: (1) control, (2) 1 mM, (3) 1.3 mM.

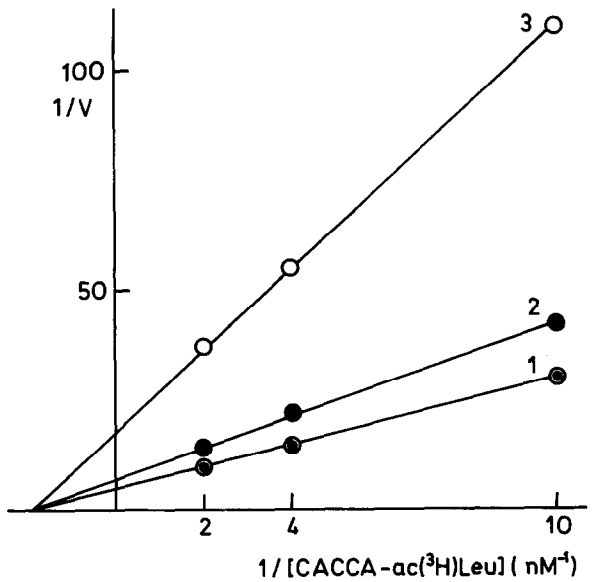

Fig.4. Inhibition of peptidyl transferase by *m*-nitrophenylboric acid at different concentrations of CACCA-ac-[^{3}H]Leu. Peptidyl transferase activity was determined in fragment reaction with 59 pmol ribosomes, 50 nmol puromycin and 0.1, 0.25 and 0.5 pmol CACCA-ac-[^{3}H]Leu; v is expressed as fmol acetylleucyl-puromycin formed min^{-1} pmol ribosomes^{-1}. The concentrations of *m*-nitrophenylboric acid: (1) control, (2) 1 mM, (3) 2 mM.

3.3. *Effect of substituted phenylboric acids on the binding of the donor and acceptor substrates to ribosomes*

A direct analysis of the effect of substituted phenylboric acids on the binding of the donor and acceptor substrates indicates that these compounds specifically inhibit the binding of the acceptor substrate, CACCA-Phe, to the acceptor binding site. The interaction of the donor substrate, CACCA-acLeu, with peptidyl transferase donor site is not affected to any significant degree (table 2). The inhibitory effect with regard to the acceptor site increases in the order: boric acid < *m*-aminophenylboric acid ≦ phenylboric acid < *o*-nitrophenylboric acid < *m*-nitrophenylboric acid.

Further evidence for an action of phenylboric acid on binding of the acceptor substrate comes from a kinetic study of inhibition of the binding of CACCA-Phe to ribosomes. The results presented in fig.5 as a double reciprocal plot indicate that phenylboric acid competitively inhibits the binding of the acceptor substrate. K_i for phenylboric acid is 5.2×10^{-4}.

4. Discussion

Phenylboric acid and its derivatives inhibit the peptidyl transferase by competing with the binding of the acceptor substrate. The decrease of peptide bond formation corresponds to the loss of binding capacity of the acceptor substrate. On the other hand, the binding of the donor substrate remains almost unaffected.

A similar change in catalytic and binding properties

Table 2
The effect of phenylboric acid, *m*-aminophenylboric acid, *m*-nitrophenylboric acid, *o*-nitrophenylboric acid and boric acid on the binding of CACCA-[^{3}H]Phe and CACCA-ac-[^{3}H]Leu to ribosomes

Inhibitor	Concentration (mM)	CACCA-[^{3}H]Phe (cpm)	(%)	CACCA-ac-[^{3}H]Leu (cpm)	(%)
–	–	3620	100	1515	100
Phenylboric acid	0.5	2020	56		
	1	1430	40		
	5	750	21	1439	95
	9			1320	88
m-Aminophenylboric acid	0.5	2110	59		
	1	1550	43		
	5	890	24	1318	88
	9			1454	96
m-Nitrophenylboric acid	0.5	250	7		
	1	120	3		
	5			1394	92
	9			1212	80
o-Nitrophenylboric acid	0.5	1070	30		
	1	490	14		
	5	260	7	1320	88
	9			1409	93
Boric acid	5	2497	69		
	10	2101	58		

The binding of acceptor substrate was examined with ribosomes (140 pmol) and CACCA-[^{3}H]Phe (0.4 pmol); 100% binding (3620 cpm) corresponded to 25% of CACCA-[^{3}H]Phe added to the reaction mixture. The binding of donor substrate was examined with ribosomes (140 pmol) and CACCA-ac-[^{3}H]Leu (0.33 pmol); 100% binding corresponded to 19% of CACCA-ac-[^{3}H]Leu added to the reaction mixture

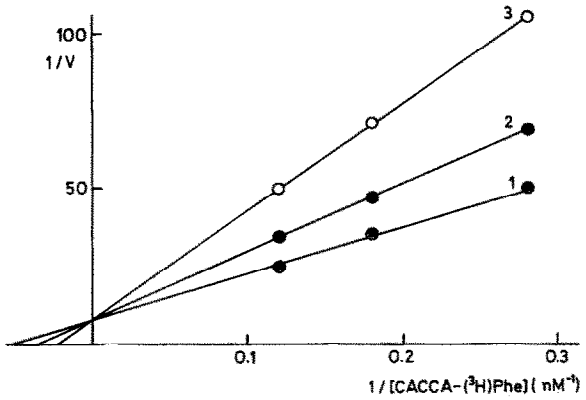

Fig.5. Inhibition of binding of CACCA-[^{3}H]Phe to ribosomes by phenylboric acid at different concentrations of [^{3}H]Phe-pentanucleotide. Binding was determined with 106 pmol ribosomes and 0.36, 0.54 and 0.81 pmol [^{3}H]Phe-pentanucleotide; v is expressed as fmol [^{3}H]Phe-pentanucleotide bound min^{-1} pmol $ribosomes^{-1}$. The concentrations of phenylboric acid: (1) control, (2) 0.3 mM, (3) 1 mM.

was observed previously after treatment of ribosomes using procedures known to modify histidine in proteins with a high degree of specificity. Photooxidation in the presence of the sensitizing dye, which modifies histidine [11], results in inactivation of peptidyl transferase [12,13]; treatment with ethoxyformic anhydride that acylates accessible histidine residues under specific conditions [14], abolishes peptidyl transferase activity of ribosomes [15]. Both these treatments affect not only peptide bond formation, but also the binding of acceptor substrate without altering the properties of the donor site [16,17].

On the basis of these observations and also in regard of the known mode of action of phenylboric acids on serine proteases [1], we are inclined to believe that a histidine residue, localized in the acceptor site of the peptidyl transferase, is involved both in the binding of the acceptor substrate and in peptide bond formation. By analogy with the proteolytic enzymes, phenylboric acid might be considered as a transition-state analogue for peptidyl transferase, which binds to its acceptor site by forming a complex with an OH-group (of protein or RNA) and a histidine residue localized at that site (fig.6). By forming this complex, phenylboric acid prevents the access of the acceptor substrate to the acceptor site of the peptidyl transferase.

The other alternative, that phenylboric acid inhibits the acceptor site of peptidyl transferase by forming a complex with the *cis*-diol group of the 3′-terminal ribose of 5 S RNA or 23 S RNA, localized incidentally in close vicinity of the acceptor site, seems less probable.

The substituent groups, which withdraw electrons from the boron atom of phenylboric acid, strengthen both the B–N and B–O interaction and also the stability of the tetrahedral adduct. The inhibitory effect of substituted phenylboric acids on peptidyl transferase increases in the expected sequence: *m*-amino- = H- < *o*-nitro- < *m*-nitro-. A similar effect of substituents was found in studies on inhibition of serine proteases by phenylboric acids [2]. This observation is also in agreement with the proposed mechanism of inhibition of peptidyl transferase by phenylboric acids.

A comparison of the effect of antibiotics or substrate analogues on peptidyl transferase and the action of phenylboric acids reveals that the effect of phenylboric acid is very similar to that of chloramphenicol. Both compounds inhibit specifically the binding of the acceptor substrate without affecting the donor site [18]. The analogy with sparsomycin and gougerotin is less pronounced, because these antibiotics also inhibit the binding of the acceptor substrate, but simultaneously stimulate the binding of the donor substrate [18].

Our interpretation of an interaction of phenylboric acid with a histidine residue localized in the acceptor site of the peptidyl transferase is in agreement with the following reports: Baxter and Zahid [15] found that inactivation of the peptidyl transferase with ethoxyformic anhydride was due to its reaction with a histidine residue in ribosomal protein L16. Protein L16 was shown to be a constituent of the acceptor site of the peptidyl transferase both by dependence of the binding of chloramphenicol to the 50 S subunit on the presence of L16 [19] and by affinity labelling [20,21]. With the use of reconstitu-

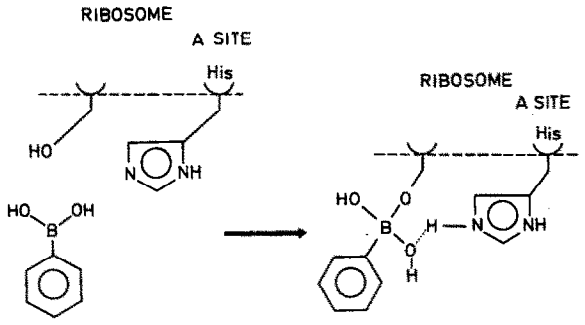

Fig.6. A tentative model of the tetrahedral adduct of phenylboric acid with the active centre of peptidyl transferase.

tion of the 50 S ribosomal subunit Dohme and Fahnestock [22] demonstrated that ribosomal proteins L2, L16 and L4 were the major targets identified for inactivation of the peptidyl transferase during photochemical modification of 50 S ribosomal subunits of *E. coli*.

Acknowledgement

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