tions were pooled and lyophilized. The residue (in 100-mg batches) was dissolved in 0.1 *M* NH_4OH, and the resulting solutions were subjected to preparative polyacrylamide gel electrophoresis using 0.1 *M* Tris–chloride buffer, pH 8.1, as the eluent. After rechromatography on AE-cellulose, the product (MTX-F) was again lyophilized and then further purified by acid precipitation from 0.1 *M* NH_4OH (178 mg, 18%): TLC on cellulose, R_f 0.71 in MeOH–NH_4OH–H_2O (7:1:2) and 0.67 in 0.25 *M* NH_4HCO_3 saturated with *n*-BuOH; absorbance max (0.1 *M* K phosphate, pH 7) 258 nm (ϵ 48,200), 365 (12,600), 495 (55,300); absorbance max (0.1 *M* NaOH) 284 nm (ϵ 36,400), 375 (12,600), 493 (67,400); fluorescence max (0.1 *M* K phosphate pH 7) excitation, 493 nm; emission, 520 nm (QY = 0.22); ^1H NMR (trifluoroacetic acid-d_1) δ 7.42 (m) and 8.46 (m) (9 H, fluorescein aromatic), 7.92 (m, 4 H, MTX aromatic), and 8.82 (s, 1 H, C-7). Anal. ($C_{46}H_{45}N_{11}O_9S \cdot 3.5H_2O$) C, H, N.

Materials and Methods. Fluorescein isothiocyanate (isomer I) and diaminopentane were obtained from Sigma Chemical Co. MTX was a gift from Dr. Florence White, National Cancer Institute; [^3H]-MTX was supplied by Dhom Products Ltd. Absorption, fluorescence, infrared, and ^1H NMR spectra were taken with Cary Model 14, Turner Model 210, Perkin-Elmer Model 337, and Jeol Model JNM-PS-100 (courtesy of Dr. J. Rivier, Salk Institute for Biological Studies) spectrometers, respectively. Quantitative polyacrylamide electrophoresis employed a Buchler Poly-Prep 200 instrument.

Dihydrofolate reductases were assayed spectrophotometrically.[14] Transport of [^3H]-MTX into *L. casei*[15] and L1210[16] cells was measured according to the indicated procedures. Enzymatic and transport inhibition constants were obtained from plots of $1/v$ against i at varying substrate concentrations where v is the initial rate of reaction or transport and i is the inhibitor concentration.

Disk electrophoresis on 7.5% (w/v) polyacrylamide gels was carried out in 0.6 × 6 cm tubes. Protein and enzymatic activity were visualized by the staining procedures of Dunlap et al.[17]

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Inhibition of Phenylalanyl-tRNA Synthetase by Aromatic Guanidines and Amidines[1]

Peter V. Danenberg

Wisconsin Clinical Cancer Center, The Medical School, University of Wisconsin, Madison, Wisconsin 53706

and Daniel V. Santi*

Department of Pharmaceutical Chemistry and Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143. *Received November 15, 1974*

Aromatic guanidines and amidines were investigated for their ability to inhibit phenylalanyl-tRNA synthetase from *E. coli* B. 2-Phenylacetamidine (**1**), benzylguanidine (**2**), and *N*-benzylbenzamidine (**3**) are competitive inhibitors with respect to phenylalanine, binding nearly as well as the substrate. The remainder of the inhibitors was unexpectedly found to be noncompetitive, indicating the presence of a secondary binding site on the enzyme. Inhibition by these compounds appears to be specific for phenylalanyl-tRNA synthetase and requires the presence of a phenyl ring as well as the amidine or guanidine moiety.

The aminoacyl-tRNA synthetases are a class of ligating enzymes that activate amino acids and attach them to the 3′ terminus of their cognate tRNA's.[2a] Each of the naturally occurring amino acids is activated with specificity by its own synthetase.[2b]

One of our objectives in a broader study of these enzymes has been to elucidate the structural features of phenylalanine critical for its recognition by phenylalanyl-tRNA synthetase and to probe the topography and localized environment of the binding site of this enzyme. By measuring the binding of a large number of substrate analogs incorporating systematic structural changes, we have been able to demonstrate that the major requirements for binding and recognition of phenylalanine analogs by phenylalanyl-tRNA synthetase are an unsubstituted phenyl ring and a protonated amino group, separated by a distance equivalent to two methylene groups.[3] The carboxyl group does not appreciably contribute to binding, and a variety of groups can be substituted into this region.

A knowledge of these factors provides the basis for de-

sign of nonclassical inhibitors[4] of enzymic reaction, which may show a greater degree of selectivity and potency than close structural analogs of substrates. In this paper we describe structure–activity relationships of a number of guanidines and amidines for inhibition of phenylalanyl-tRNA synthetase.

Experimental Section

The purified phenylalanyl-tRNA synthetase from *Escherichia coli* B and the ATP–PP_i exchange assay used here have been previously described.[3] The standard assay contained 0.3 m*M* Phe, 4 m*M* ATP, 20 m*M* $MgCl_2$, 2 m*M* $^{32}PP_i$, and limiting amounts of enzyme. Inhibition constants (K_i) were obtained by double reciprocal plots with varying phenylalanine at the aforementioned concentrations of other components. Binding of noncompetitive inhibitors is expressed as $(I/S)_{50}$ which refers to the inhibitor/phenylalanine ratio necessary to produce 50% inhibition in the standard ATP–PP_i exchange assay.

Phenylguanidine (4) was synthesized by the method of Scott et al.[5] *N,N'*-Diphenylguanidine (5) was a product of J. T. Baker Co. All of the other analogs in Table I were generously supplied by Dr. B. R. Baker. Compounds were checked for purity by analytical TLC on silica gel plates with fluorescent indicator.

Results and Discussion

Table I shows that 2-phenylacetamidine (1), benzylguanidine (2), and *N*-benzylbenzamidine (3) inhibit phenylalanyl-tRNA synthetase with K_i values similar to the K_m phenylalanine (5×10^{-5} *M*). For **2** and **3** this degree of binding is somewhat surprising since the spatial relationships of the phenyl group and the amine function differ from that of phenylalanine. The inhibition of compounds **1–3** is competitive with phenylalanine; in addition, benzylguanidine (**2**) displays noncompetitive inhibition with respect to ATP. This is to be expected on the basis of the random mechanism that we have proposed[6] for phenylalanyl-tRNA synthetase if the compound binds analogously to phenylalanine. These compounds represent the prototypes for more potent competitive inhibitors which we hope to obtain through suitable structural modification.

The remainder of the analogs in Table I was unexpectedly found to be noncompetitive with phenylalanine, apparently binding in another site on the enzyme. We suspected the presence of an allosteric binding site from previous work[3] and the binding studies performed on the phenylguanidine derivatives **4–14** serve to map this site. Since the inhibition is noncompetitive, the relative inhibitory activities of these compounds are expressed as $(I/S)_{50}$ values.

Phenylguanidine (4), which at the outset was expected to bind in the primary site due to its close resemblance to phenylalanine, was found to be a good noncompetitive inhibitor with an $(I/S)_{50}$ value of 3.5. The addition of a second phenyl group (*N,N'*-diphenylguanidine, **5**) did not affect inhibition, indicating the presence of a bulk tolerance region. Removal of one of the nitrogen atoms from **5** to give *N*-phenylbenzamidine (**6**) resulted in a tenfold binding loss. However, if **6** is compared to benzamidine (**7**), it is apparent that addition of an *N*-phenyl moiety results in a fivefold binding increase.

Placement of acetyl groups on the para or meta positions of phenylguanidine (4) to give **8** and **9** is severely detrimental to binding, resulting in 60- and 125-fold decreases. In this respect, the guanidines are similar in behavior to phenylalanine derivatives.[3] However, ortho substitution of an acetyl group (**10**) did not greatly affect binding. Interestingly, substitution on the phenyl ring of **4** with large hydrophobic groups (**11, 12, 13,** and **14**) gave inhibition which surpasses that of the parent compound. These large groups probably interact with an adjacent hydrophobic region and thus compensate for the unfavorable effect of phenyl ring substitution. Our earlier work established that the binding of phenylalanine analogs is very sensitive to substituents on the phenyl ring.[3] Therefore, the good inhibition by compounds **11–14** and especially the acetyl-substituted phenylguanidine **10** can be considered as additional evidence that there is a secondary binding site on the enzyme. Compound **12** was found to inhibit noncompetitively with respect to ATP; thus, the phenylalkoxy-substituted compounds **11–14** also do not overlap the ATP binding site.

Table I. Inhibition of ATP-$^{32}PP_i$ Exchange by Guanidines and Amidines

No.	Compd	$(I/S)_{50}$[a]	Type of inhibition[b]	K_i,[c] *M*
1	$C_6H_5CH_2C(=NH)NH_2$	8.5	Comp	2.3×10^{-4}
2	$C_6H_5CH_2NHC(=NH)NH_2$	3.0	Comp[d]	7.7×10^{-5}
3	$C_6H_5CH_2NHC(=NH)C_6H_5$	1.3	Comp	5.8×10^{-5}
4	$C_6H_5NHC(=NH)NH_2$	3.5	Noncomp	
5	$C_6H_5NHC(=NH)NHC_6H_5$	3.2	Noncomp	
6	$C_6H_5NHC(=NH)C_6H_5$	35	Noncomp	
7	$C_6H_5C(=NH)NH_2$	167		
8	*m*-$CH_3COC_6H_4NHC$-$(=NH)NH_2$	213		
9	*p*-$CH_3COC_6H_4NHC$-$(=NH)NH_2$	442		
10	*o*-$CH_3COC_6H_4NHC$-$(=NH)NH_2$	8		
11	*p*-$C_6H_5(CH)_2OC_6H_4$-$NHC(=NH)NH_2$	4.1	Noncomp	
12	*p*-$C_6H_5(CH_2)_3OC_6H_4$-$NHC(=NH)NH_2$	0.75	Noncomp[d]	
13	*m*-$C_6H_5O(CH_2)_3OC_6H_4$-$NHC(=NH)NH_2$	1.8	Noncomp	
14	*m*-$C_6H_5(CH_2)_3OC_6H_4$-$NHC(=NH)NH_2$	0.75	Noncomp	
15	$NH_2C(=NH)NH_2$	*e*		
16	*n*-$C_4H_9NH(C=NH)NH_2$	*e*		

[a] $(I/S)_{50}$ values refer to the ratio of inhibitor to substrate necessary to produce 50% inhibition. The concentration of L-phenylalanine used was 0.3 m*M* (see Experimental Section). [b] L-Phenylalanine as the variable substrate. [c] Determined from Lineweaver–Burk plots. [d] Noncompetitive with respect to ATP. [e] No inhibition observed at 12 m*M*.

The phenyl group is absolutely essential for binding in all the analogs described, since its omission as in guanidine (**15**) and *n*-butylguanidine (**16**) results in total loss of inhibitory activity. Phenylguanidine (**4**) has no effect on the valyl-tRNA synthetase from *E. coli* at [I]/[S] = 40, indicating that these compounds are not general inhibitors of aminoacyl-tRNA synthetases.

It has recently been demonstrated that phenylalanyl-tRNA synthetase has two iteractive sites which are negatively cooperative.[7,8] It is tempting to propose a mechanism of inhibition for the aromatic guanidines and amidines based on this finding. According to this model, the competitive inhibitors (**1, 2,** and **3**) would only bind to the high affinity site of the enzyme and would therefore be completely displaced by high concentrations of phenylalanine; alternatively, if they bound to the lower affinity site, their presence would have no effect on the catalytic properties of the primary site. The other inhibitors would bind to the secondary site when phenylalanine is bound to the primary site, resulting in a decrease in the maximum velocity and the observed noncompetitive inhibition. Should this interpretation be correct, the noncompetitive inhibitors described here would provide important tools for investigations of cooperative effects of the enzyme.

The information obtained in this study provides the

basis for future design of candidate irreversible inhibitors of phenylalanyl-tRNA synthetase. In addition to the guanidine or amidine function, these shall be characterized by (a) two phenyl rings, one for binding and the other to carry a reactive covalent-bond forming group, and (b) the structural features described here which will determine whether the inhibitor shall bind to the catalytically operative active site or a remote site of the enzyme.

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2-Phenethylimidazole Derivatives. Synthesis and Antimycotic Properties

Erik F. Godefroi,* Jack J. H. Geenen,

Department of Organic Chemistry, The University of Technology, Eindhoven, The Netherlands

Bert van Klingeren, and Leo J. van Wijngaarden

National Institute of Public Health, Bilthoven, The Netherlands.

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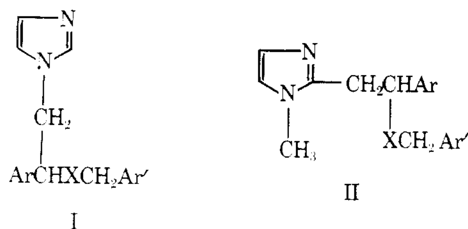
Compounds of type I (X = O, NH; Ar and Ar' = phenyl or substituted phenyl; ten examples) were prepared and assayed against miconazole (II, X = O; Ar = Ar' = 2,4-$Cl_2C_6H_3$) as potential antimycotic agents. Optimal activity was noted for I (X = O; Ar = Ar' = 2,4-$Cl_2C_6H_3$), the direct analog of miconazole. It is about one-tenth as active.

N-Substituted phenethylimidazoles of type I (X = O, NH) have been shown to display potent in vitro and in vivo antifungal properties.[1,2] One of these, namely miconazole[3] (X = O; Ar = Ar' = 2,4-$Cl_2C_3H_3$), is currently finding use in human medicine as an antimycotic agent. Continued interest in this class of compounds made us turn attention to the preparation and biological evaluation of members of type II, which bear relation to I in that the N-substituent, on having been transposed to C-2, has been replaced by a Me group. The results of these studies are herein reported.



Chemistry. Recent work from our laboratories described a facile and high-yield process for transforming 1,2-dialkylimidazoles into N-substituted 2-(2-imidazolyl)acetophenones;[4] the method was therefore utilized to prepare ketones **2a,b**. To this end 1,2-dimethylimidazole (DMI) was treated respectively with *p*-chloro- and 2,4-dichlorobenzoyl chloride in Et_3N-containing MeCN to give enol esters **1a,b**; these were then hydrolyzed to **2a,b**. In its respective reactions with *p*-chloro- and 2,4-dichlorobenzylamine, ketone **2b** furnished solid condensation products in high yields. These were assigned enamine structures **3a,b** rather than those of the corresponding imines on the basis of vinyl proton signals at δ 5.01. Failure of $NaBH_4$ to bring about reduction of these enamines was therefore not surprising. Recourse was then taken to the use of $NaBH_3CN$, a reagent recently shown to be effective in reducing enamines under acidic conditions.[5] Under related circumstances **3a,b** were readily transformed into amines **4a,b** (Scheme I).

$NaBH_4$ reduction of ketones **2a,b** led to carbinols **5a,b**. The latter may formally be considered as stemming from the addition of DMI to aromatic aldehydes and should, in principle, also be accessible in one step from these components. 1,2-Dialkylimidazoles, however, tend to undergo electrophilic processes at C-5. This was shown to be so for hydroxymethylation[6] and also for the BuLi-induced lithiation as demonstrated by isolation of **6** on further treatment of the mixture with C_6H_5CHO.[7] In our group, treatment of DMI successively with C_6H_5Li and C_6H_5CHO gave approximately a 50:50 mixture of **6** and **5c** as shown by NMR data[8] (see Experimental Section). Even though these could be chromatographically separated, the method, not lending itself for indefinite scale up, was abandoned.

Alcohols **5a,b** were anionized (NaH–THF) and were subsequently treated with the appropriate benzyl chlorides to give ethers **7a–h**; these were isolated and assayed as nitrate salts (Table I).

Experimental Section

Chemistry. Melting points, taken on a Mettler FP1 apparatus, are uncorrected. Analytical samples had ir and NMR spectra compatible with assigned structures. Combustion data for C, H, and N, obtained by Messrs. P. van den Bosch and H. Eding of these laboratories, were within 0.4% of theory.

Compound 1b. To a solution of 43.2 g (0.45 mol) of DMI and 100 g (1.00 mol) of Et_3N in 400 ml of MeCN was added dropwise and with stirring below 10° 207 g (1.00 mol) of 2,4-Cl_2BzCl. After 1 hr at room temperature, 3 l. of H_2O and 1 l. of Et_2O were added to give, on filtration and recrystallization (C_6H_6–*i*-Pr_2O), 110 g (55%) of **1b**, mp 136–139°. Anal. ($C_{19}H_{12}Cl_4N_2O_2$) C, H, N.

2',4'-Dichloro-2-[2-(1-methylimidazolyl)]acetophenone (2b). A solution of 67 g (0.15 mol) of **1b** in 450 ml of a 2:1 mixture of AcOH–HCl was refluxed for 3 hr. Solvent removal and repeated trituration of the residue with Me_2CO yielded, on filtration, 36.5 g (78%) of product hydrochloride. A small sample was recrystallized from *i*-PrOH–*i*-Pr_2O: mp 206–207°. Anal. ($C_{12}H_{10}Cl_2N_2O \cdot HCl$) C, H, N.

The main batch, taken up in H_2O and treated with $NaHCO_3$, gave crude, solid **2b**. It was dissolved in CH_2Cl_2 which was washed, dried, and evaporated, leaving **2b**, mp ~85°.