Irreversible Enzyme Inhibitors. LXXIV. Inhibitors of Guanine Deaminase. II. Studies on Bulk Tolerance within Enzyme–Inhibitor Complexes[1]

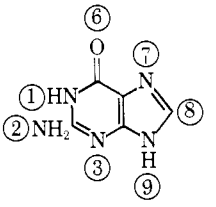
B. R. BAKER AND DANIEL V. SANTI

Department of Chemistry, University of California, Santa Barbara, California 93106, and the Department of Medicinal Chemistry, State University of New York at Buffalo, Buffalo, New York

Received August 25, 1966

8-Phenylguanine (II) was an inhibitor of guanine deaminase, but a 20-fold loss in binding, compared to substrate, occurred; even larger losses in binding occurred with the phenethyl (IV) or phenylpropyl (III) substituents on guanine. Although bulk tolerance is poor at the 8 position, an in-plane phenyl group was definitely tolerated better than the more bulky phenylalkyl groups. Although the 9-hydrogen of guanine is apparently a binding point to the enzyme, as evidenced by a 20-fold loss in binding when the 9-hydrogen is replaced by methyl, this loss could be recoupled by proper 9 substituents such as 9-phenyl; since substitution by 9-(*p*-chlorophenyl) gave even better binding, the phenyl group probably has some charge-transfer complexing character in addition to hydrophobic bonding. 9-Alkyl- and 9-aralkylguanines which have the 9 substituent out-of-plane with the purine ring were poor inhibitors. The best inhibitor of guanine deaminase to date is 9-(*p*-chlorophenyl)guanine which binds to the enzyme about fourfold better than the substrate, guanine.

In the previous paper of the series,[2] the mode of binding of guanine to guanine deaminase was studied. It was noted that the 1-hydrogen, 9-hydrogen, 7-nitrogen, and 6-oxo groups of guanine (I) were probable



I

binding points to the enzyme; the 3-nitrogen could be a binding point to the enzyme but no evidence was available. In order to design an active-site-directed irreversible inhibitor[3,4] of this enzyme, it was next necessary to determine where bulky groups could be substituted on the guanine molecule that would still allow a reasonably good reversible complex with the enzyme to be formed. Since areas 1, 3, 6, 7, and 9 appear to be binding areas to the enzyme and since area 2 is where the enzymic reaction takes place by addition of water to the 2,3 double bond, all of these five areas seemed in close proximity to the enzyme and were unlikely to tolerate large groups within the complex. This left only the 8 area for study.

8-Phenylguanine (II) was an inhibitor of the enzyme, but a 20-fold loss in binding occurred (Table I); a considerably greater loss occurred with the 8-phenethyl (IV) of the 8-phenylpropyl groups (III). Thus, the in-plane 8-phenyl group was better tolerated within the enzyme-inhibitor complex than was the more bulky phenylalkyl groups of III and IV. Although II binds sufficiently well to investigate the positioning of a covalent-forming group, such as bromoacetamido, for possible irreversible inhibitors of the isolated enzyme,[3,4] the concentration that might be necessary for *in vivo* activity would appear to be unattractively high.

Since the 8 position of guanine had only poor bulk tolerance and all the other positions on guanine appeared to be in close proximity to the enzyme within the complex, a second avenue was investigated. It is possible to remove a binding point if increased binding can be obtained elsewhere;[4] one attractive pursuit was to determine if hydrophobic bonding by alkyl, aryl, or aralkyl could be obtained with groups at the 1 or 9 positions, even though these positions appear to be binding points;[5] the 7 position was considered less feasible since two binding points might be lost. A series of 9-alkyl, -aralkyl-, and -arylguanines have been previously synthesized by Robins, *et al.*;[9] the corresponding 1 derivatives are less available. Due to the generosity of Professor Robins, we were able to investigate these 9-substituted guanines and 9-substituted 8-azaguanines as inhibitors of guanine deaminase.

9 Substituents on guanine of the noncyclic aliphatic type such as *n*-amyl (VI), *i*-amyl (VII), and 2-methylbutyl (VIII) gave little change in binding compared to 9-methylguanine (V); similarly, 9-benzyl (X) and 9-(2-furylmethyl) (XII) showed no increment in binding over 9-methylguanine (V). 9-Cyclohexylguanine (IX) showed a relatively small twofold increment in binding over 9-methylguanine (V). In contrast, 9-phenylguanine (XIII) was an excellent inhibitor of the enzyme, being complexed slightly better than the substrate, guanine, and 28-fold better than 9-methylguanine (V); that is, the binding lost by removal of the 9-hydrogen of guanine was regained with a 9-phenyl substituent. The binding was further tightened 2.6-fold by addition of a *p*-chloro substituent (XIV) on the 9-phenyl moiety, thus indicating that some charge-transfer character may be present in addition to hydrophobic bonding.

The lack of binding by 9-alkyl groups indicates that a flat interaction of the 9-phenyl group of XIII is necessary for binding. A flat interaction could be given by an aryl group on the enzyme such as phenyl or indolyl;

(1) This work was generously supported through Grants CA-05867 and CA-08695 from the National Cancer Institute, U. S. Public Health Service.

(2) For the previous paper of this series, see B. R. Baker, *J. Med. Chem.*, **10**, 59 (1967).

(3) B. R. Baker, *J. Pharm. Sci.*, **53**, 347 (1964), a review.

(4) B. R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibitors. The Organic Chemistry of the Enzymic Active Site," John Wiley and Sons, Inc., New York, N. Y., in press.

(5) The use of hydrophobic bonding to a nonfunctional area on dihydrofolic reductase has been previously described.[4,6-8]

(6) B. R. Baker and B.-T. Ho, *J. Heterocyclic Chem.*, **2**, 335 (1965).

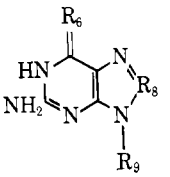
(7) B. R. Baker and B.-T. Ho, *J. Pharm. Sci.*, **55**, 470 (1966).

(8) B. R. Baker, B.-T. Ho, and D. V. Santi, *ibid.*, **54**, 1415 (1965).

(9) R. K. Robins, *et al.*, *J. Am. Chem. Soc.*, **80**, 2751 (1958); *ibid.*, **81**, 3049 (1959); *J. Med. Pharm. Chem.*, **5**, 558 (1962).

TABLE I

INHIBITION OF GUANINE DEAMINASE BY



No.	R_6	R_8	R_9	Concn, μM	% inhib[a]	Estd $[I]/[S]_{0.5}$[b]
II	=O	C_6H_5C	H	250	50	19
III	=O	$C_6H_5(CH_2)_3C$	H	250	0	>100[c]
IV	=O	$C_6H_5(CH_2)_2$	H	175	11	~110
V	=O	CH	CH_3	275[d]	50	21
VI	=O	CH	n-C_5H_{11}	450	50	34
VII	=O	CH	i-C_5H_{11}	210	50	16
VIII	=O	CH	$CH_2CH(CH_3)C_2H_5$	200	50	15
IX	=O	CH	Cyclohexyl	125	50	9.4
X	=O	CH	$C_6H_5CH_2$	370	50	28
XI	=O	CH	p-$ClC_6H_4CH_2$	350	50	26
XII	=O	CH	2-Furfuryl	320	50	24
XIII	=O	CH	C_6H_5	10	50	0.75
XIV	=O	CH	p-ClC_6H_4	3.8	50	0.29
XV	=O	N	p-ClC_6H_4	18	50	1.4
XVI	=S	CH	C_6H_5	83	50	6.2

[a] The technical assistance of Mrs. Gail Salomon is acknowledged. Guanine deaminase (guanase) from rabbit liver was purchased from Sigma Chemical Co. and assayed with 13.3 μM guanine in 0.05 M Tris buffer (pH 7.4) diluted with 10% DMSO as previously described.[2] Inhibitors were dissolved in DMSO. [b] Ratio of concentration of inhibitor to 13.3 μM guanine giving 50% inhibition. [c] Since 15% inhibition is readily detectable, the concentration for 50% inhibition is at least 5.5 times greater than that measured. [d] No substrate properties were observed at this concentration.

such an aryl–aryl interaction could also have some charge-transfer character which could be influenced by an inductive effect of a p-chloro substituent. Further studies are underway to substantiate direct binding by the in-plane 9-phenyl group of XIII to the enzyme and perhaps shed light on the mode of binding.

9-Phenyl-6-thioguanine (XVI) is also an inhibitor of guanine deaminase, being complexed about one-eighth as effectively as 9-phenylguanine (XIII); similarly, 9-(p-chlorophenyl)-8-azaguanine (XV) complexes about one-fifth as well as 9-(p-chlorophenyl)guanine (XIV) and almost as well as guanine. These results with XV and XVI agree with the fact that thioguanine and 8-azaguanine are substrates.[2]

The fact that guanosine and guanylic acid are not substrates[10] can now be rationalized on the basis that neither can complex effectively to the enzyme since the 9-hydrogen is a direct binding point; furthermore, the loss of the 9-hydrogen also causes a loss of substrate properties.

Placement of additional substituents terminating in a bromoacetamido group on the 9-phenyl substituent of 9-phenylguanine (XIII) or 9-phenyl-8-azaguanine could afford candidate active-site-directed irreversible inhibitors[3,4] of guanine deaminase; such studies are underway.

Experimental Section[11,12]

2,6-Diamino-5-phenylbutyramido-4-pyrimidinol.—To a vigorously stirred solution of 4.8 g (20 mmoles) of 2,5,6-triamino-4-pyrimidinol sulfate in 120 ml of 2 N aqueous NaOH cooled in an ice bath was added 1.8 g (20 mmoles) of phenylbutyryl chloride; 15 min later a second 1.8-g (20 mmoles) portion of acid chloride was added. After being stirred an additional 1 hr in the ice bath, the solution was adjusted to about pH 8 with acetic acid. The product was collected on a filter and washed with water. One recrystallization from 10% aqueous acetic acid and one recrystallization from 90% 2-methoxyethanol with the aid of decolorizing carbon gave 2.7 g (47%) of analytically pure white crystals: mp 269–271° dec; λ_{max} (pH 1) 269 mμ, OD ratio 280/260 = 2.10; (pH 13) 264 mμ, OD ratio 280/260 = 4.40. The compound moved as a single spot in chloroform–ethanol (5:3) on tlc.

Anal. Calcd for $C_{14}H_{17}N_5O_2 \cdot 0.67H_2O$: C, 56.2; H, 6.15; N, 23.4. Found: C, 56.5; H, 6.31; N, 23.2.

2,6-Diamino-5-phenylpropionamido-4-pyrimidinol was prepared in the same fashion as the above higher homolog; yield, 47% of analytically pure material: mp 298–300°; λ_{max} (pH 1) 267 mμ OD ratio 280/260 = 2.11; (pH 13) 264 mμ, OD ratio 280/260 = 4.06.

Anal. Calcd for $C_{13}H_{15}N_5O_2$: C, 57.1; H, 5.53; N, 25.6. Found: C, 56.9; H, 5.52; N, 25.4.

8-Phenethylguanine (IV).—A suspension of 1.40 g (5.1 mmoles) of 2,6-diamino-5-phenylpropionamido-4-pyrimidinol in 30 ml $POCl_3$ was refluxed until solution was complete (about 4 hr). The excess reagent was removed by spin evaporation *in vacuo*. Trituration of the residue with about 100 g of crushed ice gave a yellow solid that was collected on a filter and washed with water; yield, 1.15 g of crude 2-amino-6-chloro-8-phenethylpurine: λ_{max} (pH 1) 241, 314 mμ; (pH 13) 228, 311 mμ.

The crude chloropurine was refluxed with 90 ml of 2 N HCl for 3.5 hr. After clarification by filtration, the solution was brought to neutrality with NH_4OH. The product was collected on a filter and washed with water; yield, 0.65 g (50%) of an off-white powder, mp >300°, that moved as a single spot in chloroform–ethanol (5:3). Recrystallization from aqueous 2-methoxyethanol with the aid of decolorizing carbon gave white crystals: λ_{max} (pH 1) 253, 283 mμ; (pH 13) 278 mμ.

Anal. Calcd for $C_{13}H_{13}N_5O$: C, 61.2; H, 5.13; N, 27.4. Found: C, 61.4; H, 5.30; N, 27.3.

(10) A. Roush and E. R. Norris, *Arch. Biochem.*, **29**, 124 (1950).

(11) A more detailed description of the development of these methods for III and related compounds will be described in a forthcoming paper on inhibition of dihydrofolic reductase by substituted 2,6-diaminopurines.

(12) Melting points were determined in capillary tubes on a Mel-Temp block and those below 230° are corrected. Ultraviolet spectra were determined in water with a Perkin-Elmer 202 spectrophotometer. Thin layer chromatography (tlc) was done on Brinkmann silica gel GF and spots were located by visual examination under ultraviolet light.

8-Phenylpropylguanine (III) was prepared in the same manner as IV; yield 54% of product that moved as a single spot on tlc in chloroform–ethanol (5:3). For analysis a sample was dissolved in 1.5 *N* NH_4OH and reprecipitated with glacial acetic acid: mp >250°; λ_{max} (pH 1) 253, 283 mμ; (pH 13) 278 mμ.

Anal. Calcd for $C_{14}H_{15}N_5O$: C, 62.4; H, 5.61; N, 26.0. Found: C, 62.2; H, 5.75; N, 26.0.

8-Phenylguanine (II) was prepared by the literature method[12] by treatment of 5-benzamido-2,6-diamino-4-pyrimidinol with $POCl_3$. The compound has λ_{max} (pH 2) 258, 268, 305 mμ; (pH 12) 238, 312 mμ, in agreement with those reported.[13] Ring closure with polyphosphoric acid at 150°[14] for 1.5 hr was a more consistent method than $POCl_3$ and gave a 60% yield of pure material. The polyphosphoric acid was not successful for preparation of III and IV.

(12) G. B. Elion, E. Burgi, and G. H. Hitchings, *J. Am. Chem. Soc.*, **73**, 5235 (1951).

(13) S. C. Cheo, E. Chinoporos, and H. Terzian, *J. Org. Chem.*, **30**, 1946 (1965).

Experimentally Induced Phenylketonuria. 1. Inhibitors of Phenylalanine Hydroxylase

JOSEPH I. DEGRAW, MICHAEL CORY, W. A. SKINNER, MYNA C. THEISEN, AND CHOZO MITOMA

Departments of Pharmaceutical Chemistry and Biochemistry, Stanford Research Institute, Menlo Park, California

Received September 6, 1966

The action on phenylalanine hydroxylase of a series of *o*-dihydroxy aromatic compounds and some substituted phenylalanine derivatives was studied. Chemical syntheses are reported for 3,4-difluoro-, 3-chloro-4-fluoro-, 3-bromo-4-fluoro-, 4-fluoro-α-methyl-, and 4-methyl-α-methylphenylalanine. The results of inhibition studies of phenylalanine hydroxylase confirm that 4-fluorophenylalanine is a good inhibitor. Introduction of a group larger than fluorine at the 4 position reduces the inhibition. Substitution about the amino acid moiety seems to be either without significant effect or detrimental to activity. Several *o*-dihydroxy compounds were inhibitors, but esculetin (6,7-dihydroxycoumarin) was the strongest inhibitor found.

Phenylketonuria (PKU) is a heritable metabolic disorder characterized by high plasma phenylalanine (PA), urinary excretion of phenyl ketones, and mental deficiency. The basic metabolic aberration lies in the inability of the diseased individual to oxidize PA to tyrosine. PA hydroxylase is known to be inhibited by a high concentration of PA, its own substrate,[1] thus the diseased state may be induced by a PA-enriched diet. A compound that can act as a specific inhibitor of PA hydroxylase would be desirable in studying experimental PKU. The disease state produced by such a compound not only would resemble PKU in its etiology, but would also be free of complications arising from the presently employed high PA diets.

Two structurally unrelated compounds, 4-fluorophenylalanine[2] and esculetin (6,7-dihydroxycoumarin)[3] are known to be reversible inhibitors of PA hydroxylase. The latter, by our observations, was about 20 times as potent as the former in this regard and was chosen as a starting point for our work. The 6- and 7-methoxy derivatives and dihydroesculetin were examined. In addition some other *o*-dihydroxy aromatic compounds were evaluated. A second broad class of potential inhibitors was comprised of substituted PA compounds. Various substituents were placed on the aromatic ring and some compounds were prepared with alterations about the amino acid portion of the PA molecule.

Enzyme Inhibition Studies.—Phenylalanine hydroxylase was prepared from rat liver by the method of Kaufman.[4] Purification was carried out up to step 2 of this method. The incubation mixture consisted of 100 μmoles of sodium phosphate buffer, pH 7.4, 20 μmoles of reduced nicotinamide–adenine dinucleotide, 10 μmoles of nicotinamide, approximately 10 mg of enzyme protein, 1.0 μmole of phenylalanine, and appropriate amounts of the test compound in a final volume of 2 ml. Incubation was carried out for 20 min at 37° in air. Tyrosine was assayed by the method of Udenfriend and Cooper.[5]

Biological Results and Discussion

The compounds tested as inhibitors of PA hydroxylase are listed in a decreasing order of potency in Table I. In general, *o*-dihydroxy-type compounds were potent inhibitors in agreement with the findings of Burkard, *et al.*,[6] and Ross and Haljasmaa.[3] As was pointed out by Fuller[7] in his inhibition studies on tryptophan hydroxylase, which may be identical with PA hydroxylase,[8] the inhibitory property of esculetin depended on the *o*-dihydroxy structure. It was inferred[7] that metal chelation by the *o*-dihydroxy moiety was responsible for the inhibition. In our studies 6-methyl-, 6-glucosyl-, and 7-methylesculetin showed considerably weaker inhibitory activity than esculetin. Also, the 3,4 double bond of the coumarin nucleus of esculetin is apparently required as evidenced by the greatly reduced potency of 3,4-dihydroesculetin as an inhibitor.

Udenfriend, *et al.*,[9] showed that 3,4-dihydroxyphenyl-α-propylacetamide inhibited tyrosine hydroxylase by competing with the cofactor, tetrahydropteridines. Since PA hydroxylase activity also is dependent on reduced pteridines,[10] the mechanism by which esculetin inhibits PA hydroxylase may be similar.

(1) S. Udenfriend and J. R. Cooper, *J. Biol. Chem.*, **194**, 503 (1952).
(2) D. D. Watt and J. P. Vandervoorde, *Federation Proc.*, **23**, 146 (1964).
(3) S. B. Ross and O. Haljasmaa, *Life Sci.*, **3**, 579 (1964).
(4) S. Kaufman, *Methods Enzymol.*, **5**, 809 (1962).
(5) S. Udenfriend and J. R. Cooper, *J. Biol. Chem.*, **196**, 227 (1952).
(6) W. P. Burkard, K. F. Gey, and A. Pletscher, *Life Sci.*, **3**, 27 (1964).
(7) R. W. Fuller, *ibid.*, **4**, 1 (1965).
(8) J. Renson, H. Weissbach, and S. Udenfriend, *Biochem. Biophys. Res. Commun.*, **6**, 20 (1962).
(9) S. Udenfriend, P. Zaltzman-Nirenberg, and T. Nagatsu, *Biochem. Pharmacol.*, **14**, 837 (1965).
(10) S. Kaufman, "Oxygenases," O. Hayaishi, Ed., Academic Press Inc., New York, N. Y., 1962, p 129.