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A DIAZINE HETEROCYCLE REPLACES A SIX-MEMBERED HYDROGEN-BONDED ARRAY IN THE ACTIVE SITE OF SCYTALONE DEHYDRATASE

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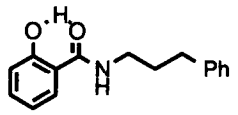
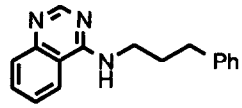
Abstract: 4-aminoquinazoline **II** is a fully functional substitute for salicylamide **I** as a competitive inhibitor of scytalone dehydratase. Kinetic measurements demonstrate that both inhibitors bind to the enzyme in their neutral form, suggesting that the hydrogen-bonded array in I binds to the enzyme active site as an intact structural element.

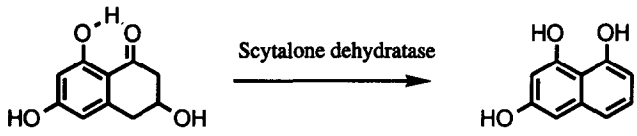


I



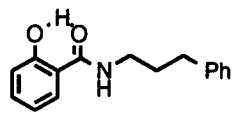
II

Scytalone dehydratase (SD)[1] catalyzes the conversion of scytalone to 1,3,8 tri-hydroxynaphthalene in the melanin biosynthesis pathway of several fungal species. Inhibition of this pathway is of practical importance in agriculture as exemplified by the control of the rice blast fungus, *Magnaporthe grisea*, by melanin biosynthesis inhibitors[2] that act at other sites in the pathway. Since *M. grisea* mutants deficient in SD have been shown to be unable to infect rice plants[3], successful chemical interruption at the SD step might provide novel, highly specific agents for the prevention of rice blast.



Scytalone 1,3,8-trihydroxynaphthalene

Our initial efforts to design SD inhibitors, based on knowledge only of substrate and product structure and an assumed reaction mechanism, revealed that the *peri*-hydroxyl carbonyl moiety present in the substrate was important for tight binding. We discovered competitive inhibitors with K_i values in the low nanomolar range, such as salicylamide **I** , but deletion of either the phenol or the carbonyl oxygen dramatically reduced affinity. In addition, the amide residue appeared to be required for high affinity.[4]



I

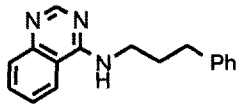
Several observations suggested that **I** binds to SD as an intact, cyclic hydrogen-bonded array, rather than contributing a pair of hydrogen bond acceptors or a donor/acceptor pair. First, inhibitors lost activity by more than two orders of magnitude if fluorine, amino, acetamido or methoxy replaced hydroxyl; this result shows that an electron lone pair at the *ortho*-position, in any of several orientations, is insufficient to explain the observed affinity. Second, the affinity of an inhibitor such as **I** decreased with increasing pH whereas the enzyme showed little change in its maximum rate of reaction with substrate over the pH range 6 to 10 (not shown). The protonated (neutral) form of **I** bound at least 30-fold more tightly to SD than did the deprotonated form (Table).[5]

Table

Compound	pK[6]	K_i, μM[7]	
		Protonated	Deprotonated
I	8.1	0.0073 (pH 7.0, neutral)	0.23 (pH 9.8, anion)[5]
II	6.1	1.8 (pH 5.5, cation)[8]	0.019 (pH 8.8, neutral)
III	9.0	88 (pH 7.0, cation)	45 (pH 9.8, neutral)
IV	7.5	6.7 (pH 6.0, cation)	2.3 (pH 9.8, neutral)

Finally, ^{1}H NMR of all *ortho*-hydroxybenzoyl inhibitors used in this study indicated a strong hydrogen bond between the two oxygens, as expected[9].

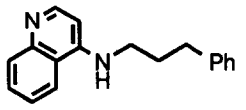
These observations, and the need to stabilize the salicylamides to metabolic deactivation[10], led us to replace the array with the amidine present in quinazoline **II**[11]. If the binding model were correct, that is, if the neutral H-bonded six-membered ring were recognized as an intact structural entity, then the diazine ring in **II** might be expected to bind to SD with comparable affinity to **I**.



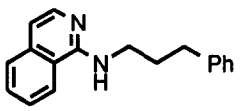
II

In fact, **I** and **II** inhibited the enzyme with similar potencies at neutral pH. Measurement of the dependence of inhibition on pH revealed that the neutral forms of **I** and **II** were the most potent inhibitors (Table). That is, regardless of the intrinsic pK values of the inhibitors, or whether the inhibitors equilibrated between neutral

and negatively charged forms or neutral and positively charged forms, the neutral form bound most tightly to the enzyme. This observation also held for analogs **III** and **IV**[11], although they were weaker inhibitors of the enzyme even in the neutral form (Table). Apparently, the active site of SD is designed for optimal binding of neutral species, an observation further supported by our finding that even neutral inhibitors and substrates bind more poorly to SD under conditions (pH <6) in which kinetically important amino acid residues in SD become protonated.[8]



III



IV

Structural variations on salicylamide **I** that changed its affinity to SD consistently caused similar changes in binding efficacy when applied to quinazoline **II**[4], supporting the assumption that the quinazoline and the salicyl arrays bind in a similar orientation.

These results suggest that *internally* hydrogen-bonded arrays can be recognized by proteins as intact structural elements. Modifications such as the isosteric replacement of the peri-hydroxybenzoyl group with the quinazoline heterocycle described above may find use in inhibitor design strategies which require modulating the metabolic lifetime of protein ligands while retaining the intrinsic structural features required for molecular recognition. This approach could complement the more widely utilized strategy of mapping hydrogen-bond donors and acceptors directly onto heterocycles or other rigid, metabolically stable scaffolds.[12]

[1] Tajima, S.; Kubo, Y.; Furusawa, I.; Shishiyama, J. *Exptl. Mycology* **1989**, 13, 69-76. Woloshuk, C.P.; Sisler, H.D.; Tokousbalides, M.C.; Dutky, S.R. *Pestic. Biochem. Physiol.* **1980**, 14, 256-264.

[2] Chida, T.; Sisler, H.D. *Pestic. Biochem. Physiol.* **1987**, 29, 244-251.

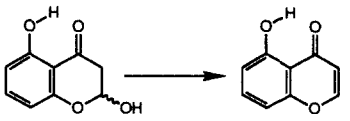
[3] Chumley, F.G.; Valent, B., *Mol. Plant-Microbe Interact.* **1990**, 3(3), 135-143.

[4] Full details concerning the structure-activity relationships of these and other inhibitors of scytalone dehydratase will be published elsewhere.

[5] If there are large differences in the intrinsic affinity of charged and neutral forms of the inhibitor, the observed binding constant may contain substantial contributions from binding of the most avid form, even if it is present in only residual amounts. For two forms of an inhibitor competitively interacting with the enzyme, $K_{i,obs} = K_a K_b(1+10^{\Delta})/(K_b + K_a \cdot 10^{\Delta})$, where Δ = pH - pK and K_a and K_b are the intrinsic binding constants of the acidic and basic forms of the inhibitor, respectively. For the interaction of **I** with the enzyme, one may calculate $K_b = 0.6$ μM when K_a ~0.0073 μM. However, given experimental error and the asymptotic relationship above, K_b may in fact be much larger (but not much smaller) relative to K_a. For example, $K_b \geq 100$ μM for $K_{i,obs} = 0.37$ μM. Given the constraints in accessible pH ranges, it is difficult in practice to distinguish between the observed 30 fold difference in binding and a much larger difference.

[6] pK values (+/- 0.15) were determined by observing the spectral changes that occurred upon titration of the protonated forms of the compounds to the deprotonated forms. Clear isosbestics were observed, and the pK values were determined by simultaneously fitting the absorbance data derived from observations at two wavelengths to the equation $[e_a + e_b 10^{(pH-pK)}]/[1+ 10^{(pH-pK)}]$, where e_a and e_b are the absorbances at the observed wavelengths of the protonated and deprotonated forms, respectively.

[7] SD activity (isolated from *Magnaporthe grisea*, maximum activity = 220 μmol/min-mg protein with scytalone; 1300 μmol/min-mg protein with **V**) was determined spectrophotometrically at various pH values by observing the rise in absorbance at 320 nm due to the conversion of **V** to **VI**
(Δe_{320} = 1.5 $mM^{-1} \cdot cm^{-1}$; see below).



V **VI**

This conversion by SD is experimentally convenient, as the spectra of these compounds do not vary with pH in the pH ranges used in this study, and the V_{max} and K_m values of SD for **V** are also invariant from pH 6 to 10, which covers most of the range needed for this study (but see footnote 8).

Three different substrate and inhibitor concentrations were used for each measurement. Competitive inhibition was observed and inhibition constants were determined by non-linear regression of the data to the equation $v(S,I) = V_mS/[K_m(1+I/K_i) + S]$. K_i values are accurate to within 25%.

V has not been described in the literature: briefly, we prepared **V** by the condensation of 2,6-dihydroxyacetophenone and ethyl formate in the presence of excess sodium hydride in diethyl ether. The dehydration product **VI** is known to be formed during treatment of the same reagents under acidic conditions (see: Becket, G. J. P.; Ellis, Gwynn P.; Trindade, M.; Iolanda U. *J. Chem. Res. (S)* **1978**, (2), 47.) . Under the basic conditions described here, primarily hydrated material is isolated although small amounts of **VI** are removed by chromatography. NMR studies indicate that the hemiacetal form predominates in aqueous and organic solvent. Deuterium readily exchanges onto the aliphatic carbon at neutral pH, however, suggesting rapid equilibration with the ring-opened aldehyde.

[8] Apparent K_m and K_i values for neutral substrates and ligands (such as scytalone, **V**, and **I**) increase ~10-fold for each unit decrease in pH below pH 6 due to protonation of an amino acid residue on SD with a pK of ~6.0 (not shown). One would expect this behavior if the protonated enzyme form had substantially less avidity for these ligands. Since the pK of **II** is 6.1, it is difficult to adjust conditions to accurately measure the intrinsic binding of protonated **II** to the ionic form of SD that exists above pH 6.0. Accordingly, the decrease in affinity observed for **II** at pH 5.5 was due in part to protonation of the enzyme and in part to protonation of the substrate. Note that at pH 5.0, K_i(**II**) > 300 μM, confirming the poor binding of **II** to the protonated enzyme.

[9] All of the inhibitors and substrates with this array show the H-bonded phenolic ^1H NMR resonance between 11 and 13 ppm in $CDCl_3$ at room temperature. The strength of this hydrogen bond has been shown to correlate with ^1H chemical shift: see Kondo, M.; *Bull. Chem. Soc. Jpn.* **1979**, 52(2), 521-3.

[10] Analytical HPLC studies of salicylamides in the presence of rice plant tissue demonstrated very short half-lives. Major routes of degradation are thought to be amide cleavage and glucuronidation of the phenol (data not shown).

[11] 4-Aminoquinazolines (**II**) were prepared by the condensation of 4-chloroquinazoline with primary amine as described in: (a) Kochergin, P. M.; Mazur, I. D.; Sinyak, R. S, U.S.S.R. From: *Otkrytiya, Izobret.*, Prom. Obraztsy, Tovarnye Znaki **1974**, 51(38), 56; (b) U.S. 4,213,987; (c) DE 3954987; (d) FR 1528020. 2- and 4-aminoisoquinolines (**III** and **IV**) were similarly prepared by the condensation of the appropriate chloroheterocycle with primary amine in the presence of a tertiary amine base.

[12] See (a) Sweet, F.; Boyd, J.; Medina, O.; Konderski, L.; Murdock, G. L.; *Biochem. Biophys. Res. Commun.*, **1991**, 180(2), 1057-63; Rosenberg, S. H.; Dellaria, J. F.; Kempf, D. J.; Hutchins, C. W.; Woods, K. W.; Maki, R. G.; De Lara, E.; Spina, K. P.; Stein, H.; et al. *J. Med. Chem.*, **1990**, 33(6), 1582-90; Muehldorf, A.V.; Van Engen, D.; Warner, J. C.; Hamilton, A. D. *J. Am. Chem. Soc.*, **1988**, 110(19), 6561-2; (d) Rebek, J.; Nemeth, D., *J. Am. Chem. Soc.*, **1986**,108(18), 5637-8.