Synthesis and Structure-Activity Studies of a Series of [(Hydroxybenzyl)amino]salicylates as Inhibitors of EGF Receptor-Associated Tyrosine Kinase Activity

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The synthesis and structure-activity relationships of a series of [(hydroxybenzylidene)amino]-salicylates and a series of [(hydroxybenzyl)amino]salicylates as inhibitors of EGF receptor-associated tyrosine kinase activity are described. Their inhibitory potency was evaluated in vitro using ER 22 cell membranes (CCL 39 cells transfected with EGF receptor) as an enzyme source and the tridecapeptide RRSrc (RRLIEDAEYAARG) as substrate. Their cellular activity was measured by inhibition of the EGF-stimulated DNA synthesis of ER 22 cells. Chemical modifications were made to analyze the role of the different substituents. The amino series was found to be more active than the imino series. The hydroquinone moiety appears to be essential for tyrosine kinase inhibitory activity in the series of 5-[(2,5-dihydroxybenzyl)amino]salicylates. Comparison of the imino and amino series by molecular modeling techniques provides further evidence in support of the hypothesis that the important reduced linking chain, CH_2NH, allows the correct positioning of the 2,5-dihydroxybenzyl ring, possibly in a cis-like conformational arrangement.

Introduction

Tyrosine kinase activity is often overexpressed in cancers[1,2] and other cellular proliferative diseases.[3,4] This makes these enzymes potential targets for new therapeutic agents. Protein tyrosine kinases catalyze the direct transfer of the γ-phosphate group from ATP to a tyrosine moiety of a substrate protein. Accordingly, compounds able to compete with either ATP[5-10] or with the substrate[11-14] as well as bisubstrate-derived molecules acting as transition state analogues have been designed as inhibitors of tyrosine kinase activity.[15-18]

Most of the inhibitors reported to compete with the substrate are tyrosine analogs, such as erbstatin or tyrphostins.[11-13] They have been shown to have antiproliferative activity on cellular tumors[19,20] and to be selective tyrosine kinase inhibitors in vitro,[21,22] although they were recently reported to block protein-serine kinases[23] and to inhibit the meiotic maturation of sea star oocytes.[24]

Since the ATP binding site is common to the different kinases, it was suspected that tyrosine kinases inhibitors which compete with ATP and are noncompetitive with the substrate would be nonselective. However, most of them, such as isoflavone derivatives, have been shown to be not only highly active but generally selective for tyrosine kinases as compared to serine/threonine kinases.[6,10] As recently discussed by Levitzki[25] and Burke,[26] this suggests that knowing whether an inhibitor competes with ATP or substrate is not sufficient to predict its selectivity.

Lavendustin A derivatives[7] isolated from *Streptomyces griseolavendus* have been described as potent tyrosine kinase inhibitors endowed with antiproliferative activity. Umezawa proposed that Lavendustin A acts as a competitive inhibitor against ATP and noncompetitive inhibitor against the peptide substrate.[7] However the complex kinetic behavior of Lavendustin A could be interpreted as a uncompetitive mechanism.[27]

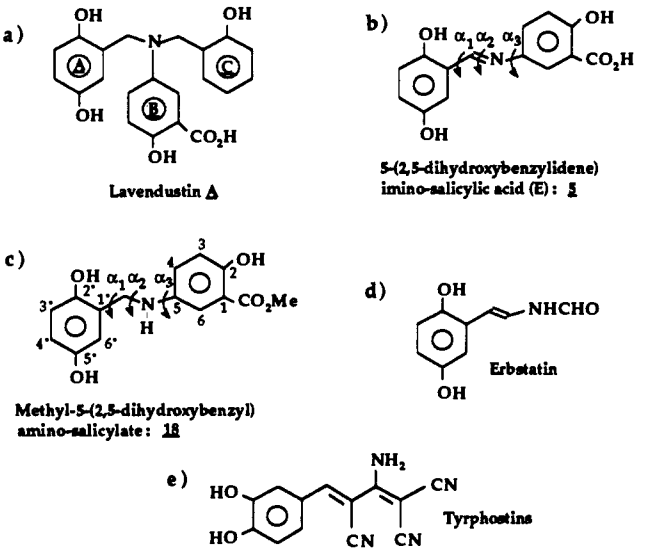

Figure 1.

Lavendustin A is composed of three aromatic rings (Figure 1), with cycles A and C bearing hydroxyl groups linked by a methyleneamino chain to the salicylate ring. In the present work, we have investigated the relative importance of the different structural parameters for the biological activity of Lavendustin and have used molecular modeling techniques to explore the conformational aspects of a representative compound of the amino series. Since cycle C does not appear to be essential for inhibition of tyrosine kinase activity,[7] a series of derivatives were synthesized bearing only the two cycles A and B, in which hydroxyl groups were successively suppressed, displaced, or exchanged by a methoxy group. The OH and COOH groups of the salicylate moiety position were inverted. The tyrosine kinase inhibitory potency of these compounds was measured in vitro using ER 22 cell membranes as an

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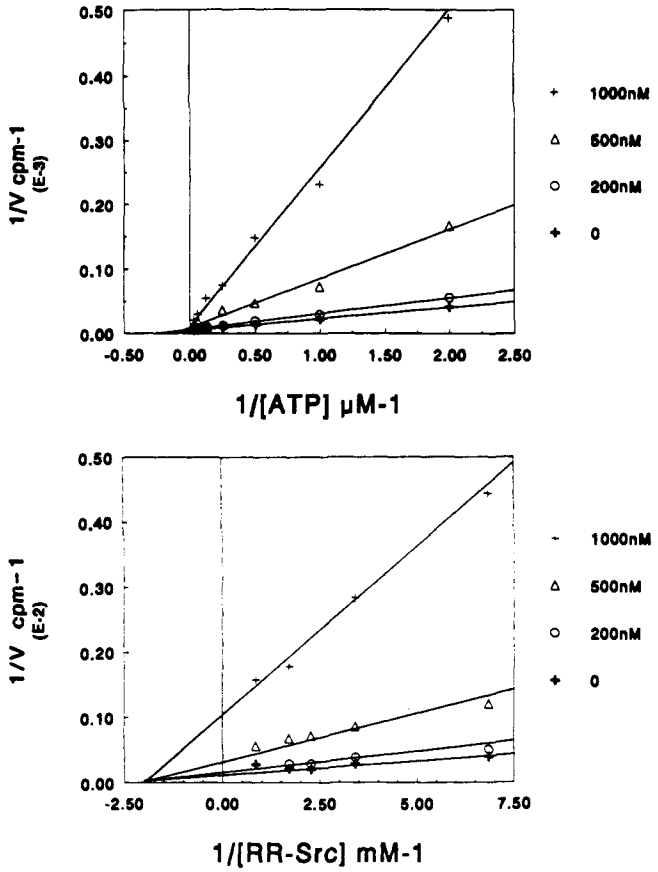


Figure 2. Lineweaver–Burk plot of the tyrosine kinase assay was performed with the peptide RRSrc as substrate. The inhibitor 18 was incubated at the indicated concentrations in the presence of varying concentrations of ATP or RRSrc. Lineweaver–Burk plot of the tyrosine kinase shows that 18 is competitive with ATP and noncompetitive with RR-Src.

enzyme source,[7] and their ability to inhibit cell growth was determined by measuring their effects on the EGF-stimulated DNA synthesis of ER 22 cells.[28,29]

Chemistry

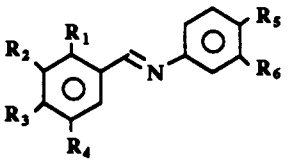
Compounds 1–12 were prepared by condensation of the corresponding hydroxy-, dihydroxy- or dimethoxybenzaldehyde with the appropriate aminosalicylate or aminobenzoate. No evidence for the formation of *E–Z* isomers mixture was noted, only the *E* isomers being obtained. The methyl aminosalicylates were obtained by esterification of the corresponding aminosalicylic acid in methanol with $SOCl_2$, except for the preparation of compound 10, where the amino group of the 5-aminosalicyclic acid was protected by Boc_2O before action of dimethylsulfate in the presence of phase-transfer catalyst followed by deprotection with TFA to yield the methyl 2-methoxy-5-aminobenzoate. Compounds 1–12 were then reduced by catalytic hydrogenation on Pd/C in methanol at room temperature and atmospheric pressure, giving the corresponding compounds 13–23. ^1H NMR and microanalyses were in agreement with the assigned structures.

Kinetics of in Vitro Binding. The EGF receptor tyrosine kinase has been reported to act in a sequential ordered bi–bi mechanism with the peptide substrate binding to the active site first followed by ATP.[30] We therefore performed a competitive kinetic analysis of 18, one of the most active compounds, against both ATP and the peptide substrate. The analysis of the results following Lineweaver–Burk treatment (Figure 2) indicates that compound 18 is a competitive inhibitor of ATP binding and a noncompetitive inhibitor of peptide substrate binding. This shows that structure–activity relationships in the series of 5-[(2,5-dihydroxybenzyl)amino]salicylates can be interpreted in terms of an interaction between the inhibitor and the ATP binding subsite. The behavior of 18 is comparable to that of Lavendustin A, but different from that of erbstatin and tyrphostin derivatives which have been reported to be competitive inhibitors of peptide substrate binding, although recent results suggest that they might be hyperbolic mixed inhibitors with respect to both ATP and the substrate.[25]

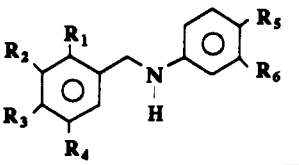
Structure–Activity Relationships. The biological results reported in Table I show that compounds of the amino series are more active than those of the imino series. This is in contrast to the structurally related erbstatin and tyrophostin derivatives where the C=C double bond conjugated with the dihydroxyphenyl ring is essential for inhibitory activity and its saturation gives less active compounds.[14] The simplest explanation is that tyrphostin and erbstatin derivatives block the peptide substrate binding site, while 5-[(2,5-dihydroxybenzyl)amino]salicylates are competitive inhibitors of the ATP binding site. Thus, the results of Table I show that while compounds of the amino series seem to interact efficiently with the ATP binding site, compounds of the imino series do not. Several reasons can be proposed to explain this different behavior, including the fact that the imino compounds are less stable chemically than the amino compounds. In addition, the nitrogen atom appears to be a hydrogen bond donor in the amino series while it has an acceptor role in the case of the imino compounds, suggesting that interactions which might be important in the binding site are fulfilled in the former but not in the latter series. Nevertheless, it is difficult to account for the very large difference in activity observed between the two series without taking conformational parameters into account. Derivatives of the imino series exist under a single form, around the C=N double bond, as shown from the unique set of NMR signals which can be attributed to the *E* form. In contrast, the derivatives of the amino series exist under a large number of conformations due to rotation around the CH_2NH bond which links the two aromatic rings. This flexibility could allow these compounds to adopt a bioactive conformation inside the active site. We therefore tried to analyze the conformational behavior of the imino and amino compounds, by modeling 6 and 18, as representative of each series, in order to appreciate the energetical relevance of conformational arrangements in each series, i.e., the location of the two aromatic rings in a cis arrangement around the CH_2–NH bond in the amino series.

Molecular Modeling. Torsions α_1, α_2, α_3 are shown in Figure 1 and defined in the Experimental Section. All 48 conformations (within 3 kcal/mol from the minimum one) of 18 and all 72 conformations of 6 were supposed to be equiprobable. Table II shows the distribution of each of three torsion angles in 6 and 18. In the case of 6, α_1 and α_3 span all values. Yet α_1 shows a preference for the 330° to 30° and for the 120° to 180° intervals. The major populations of α_3 are located in the following regions: 60°–90°, 210°–240°, and 300°–330°. In the case of 18, torsion α_3 has a higher frequency of occurrence in the neighborhood of the trans state, whereas α_1 presents a somewhat discrete distribution in the ranges 120°–150°, 210°–240° and 330°–360°, clearly avoiding the three classical rotamers ($g\pm$, t).

Table I. Activity of Tyrosine Kinase Inhibitors



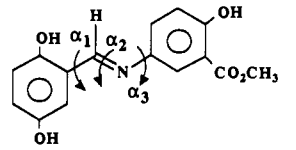
no.	R_1	R_2	R_3	R_4	R_5	R_6	mp, °C	anal.	IC_{50}, μM: inh RRSrc phosphorylation[a]	IC_{50}, μM: inh [^{3}H]TdR incorporation[b]
1	OH	H	H	H	OH	CO_2H	259–260	C,H,N	>100	>20
2	OH	H	H	H	OH	CO_2CH_3	117–118	C,H,N	≫100	≫10
3	H	OH	H	H	OH	CO_2CH_3	144–145	C,H,N	≫100	20% at 20 μM
4	H	H	OH	H	OH	CO_2CH_3	225–227	C,H,N	>10	>10
5	OH	H	H	OH	OH	CO_2H	>300 dec	C,H,N	5	>100
6	OH	H	H	OH	OH	CO_2CH_3	223–224	C,H,N	~100	
7	OH	H	H	OH	CO_2CH_3	OH	221–222	C,H,N	≫10	≫10
8	OH	H	H	OH	H	CO_2H	238–239	C,H,N	≫10	>20
9	OCH_3	H	H	OCH_3	OH	CO_2CH_3	135–136	C,H,N	≫10	14% at 10 μM
10	OH	H	H	OH	OCH_3	CO_2CH_3	206–207	C,H,N	>100	>100
11	H	OH	OH	H	OH	CO_2CH_3	206–207	C,H,N	~10	37% at 20 μM
12	OH	H	OH	H	OH	CO_2CH_3	186–187	C,H,N	>100	65% at 20 μM



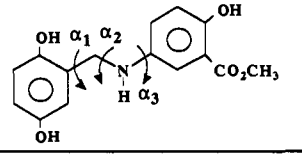
13	OH	H	H	H	OH	CO_2H	155–156	C,H,N	25	>20
14	OH	H	H	H	OH	CO_2CH_3	145.5–147.5	C,H,N	45	20
15	H	OH	H	H	OH	CO_2CH_3	139–140	C,H,N	37% at 100 μM	21
16	H	H	OH	H	OH	CO_2CH_3	135–136	C,H,N	>10	>10
17	OH	H	H	OH	OH	CO_2H	≥245 dec	C,H,N	0.03[c]	92
18	OH	H	H	OH	OH	CO_2CH_3	194–195	C,H,N	0.6	9
19	OH	H	H	OH	CO_2CH_3	OH	189–190	C,H,N	0.2	10
20	OH	H	H	OH	H	CO_2H	174–176	C,H,N	2.4	>20
21	OH	H	H	OH	H	CO_2CH_3	132–133	C,H,N	1.6	9
22	OCH_3	H	H	OCH_3	OH	CO_2CH_3	79–80	C,H,N	>100	18% at 20 μM
23	OH	H	H	OH	OCH_3	CO_2CH_3	141–142	C,H,N	5	9.5

[a] In vitro tyrosine kinase inhibitory potency was measured on ER 22 membrane fractions by competition with the RRSrc peptide, [^{32}P]γATP, 5 μM (Amersham PB 10132, 10Ci/mM), and inhibitors in various concentrations. [b] Inhibitory potency of EGF-dependent DNA synthesis was measured on ER 22 cells incubated with different concentrations of inhibitors and [^{3}H]TdR. [c] Lit.[7] IC_{50} = 0.04 μM.

Table II



a	0–30°	30–60°	60–90°	90–120°	120–150°	150–180°	180–210°	210–240°	240–270°	270–300°	300–330°	330–360°
α_1	10	8	6	5	7	9	2	5	5	2	5	8
α_3	0	3	8	6	5	4	6	9	3	5	19	4



b	0–30°	30–60°	60–90°	90–120°	120–150°	150–180°	180–210°	210–240°	240–270°	270–300°	300–330°	330–360°
α_1	4			3	16			13	1		4	7
α_2		2	5	7	5	12	2	1	2	6	4	2
α_3	4	3	1	1	8	6	11	4	1	1	4	4

[a] Cumulative statistical conformers distribution as a function of α_1 and α_3 over 30-deg intervals for compound 6 (72 conformers have been analyzed). α_2 was fixed at either 0 or 180° for the conformational search. [b] Cumulative statistical conformers distribution as a function of α_1, α_2, and α_3 over 30-deg intervals for compound 18.

The major population of α_2 lies in the 150°–180° range and two minor populations are in the ranges 90°–120° and 270°–300° (Table II). Several representative conformations for 6 and 18 are depicted in parts a and b of Figure 3, respectively.

The major populations of energetically stable conformers for 6 and 18 contain a trans arrangement around the C–N bond (α_2 = 150°–180°); Figure 3a,b shows that a larger conformational space is energetically accessible to 18 as compared to the less active compound 6. This includes a pseudo-cis arrangement around the C–N bond.

Structural Modifications. Structural modifications

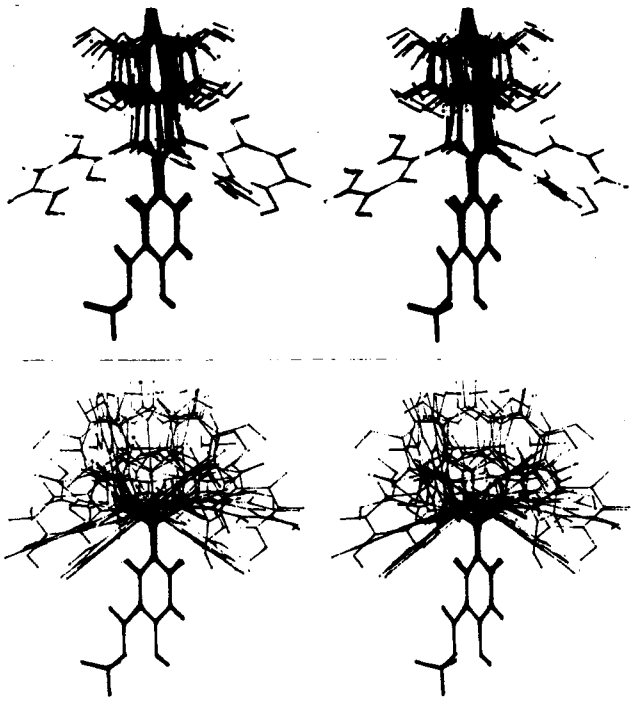


Figure 3. Stereoview of several representative conformations of compounds **6** (a) and **18** (b).

introduced on cycle A gave interesting changes in activity. Among the imino acids, only **5** is active in vitro but much less than its amino analog. Concerning the imino esters, they were relatively inactive, except compound **11**, which possesses two ortho OH groups on the A ring as in the series of Levitzki's tyrphostins (Table I). This suggests that this imino compound might mimic tyrphostins in its mechanism of action and thus might compete with the peptide substrate binding subsite in both tests. Amino compounds derived from the imino compounds **11** and **12** are unstable and could not be isolated for testing. Suppression of either one of the hydroxyl groups on ring A did not improve the activity (compounds **2**, **3**, and **4**) in the imino series. Even **4**, which possesses a 4-hydroxyphenyl ring, the substructure responsible for the potency of benzylidene malonitriles described by Levitzki,[13,14] was inactive.

In the amino series, methylation of the para hydroquinone hydroxyl groups (compound **22**) as well as suppression of either one of the two hydroxyl groups (compounds **14** and **15**) led to a large loss of activity, suggesting that the two hydroxyl groups are important for tyrosine kinase inhibition. It is interesting to note that the roles of the two hydroxyl groups are not identical, since the suppression of the OH ortho to the linker (compound **15**) resulted in a larger decrease in activity than suppression of the meta OH (compound **14**). This is hardly reconciliable with an oxido reductive pathway for the mechanism of action of these compounds.

Chemical modifications on cycle B were also performed. As previously discussed, in the imino series no compound except **5** (IC_{50} = 5 μM in vitro) was active against both tyrosine kinase activity and cellular proliferation.

In the more active amino series (Table I) modulation of the activity appears to be related to hydroxyl substitution of the phenyl rings. Compound **17**, already described by Onada et al.,[7] appears in vitro as the most active inhibitor of RRSrc phosphorylation. However, **17** did not show any significant activity against the EGF-stimulated DNA synthesis of ER 22 cells.

A similar weak cellular activity has already been observed with Lavendustin carboxylic acid derivatives, and this was attributed to the lack of cellular penetration[8] of the drug due to a too large molecular polarity. In agreement with this, methyl esterification of **17** led to the compound **18** which was less active as an enzyme inhibitor (by a factor 20) than **17**, but its inhibitory potency on EGF-dependent cellular DNA synthesis was increased by a factor 10.

The results also suggest that the salicylate moiety of these compounds might compete with ATP for chelating the Mg^{2+} ion present in the active site of the EGF receptor or might interact with the Lys^{721} residue which stabilizes ATP binding.[35] In both cases, the interaction measured in vitro would be expected to be smaller in the case of the methyl salicylates **18** and **19**, which is effectively the case, since their inhibitory potencies are IC_{50} = 0.6 and 0.2 μM, respectively. Suppression of the hydroxyl group in the salicylate moiety produces a large decrease in activity as shown by the difference between **20** (IC_{50} = 2.4 μM) and **17** (IC_{50} = 0.03 μM). This seems to be in favor of a stabilizing interaction by means of the salicylate moiety involving a coordinating effect of Mg^{2+}, instead of a simple hydrogen bonding with Lys^{721}.

Compounds **17** and **20** are weakly active against EGF-dependent cellular growth, whereas their methyl ester compounds **18** and **21** have higher activities, suggesting that the bioavailability of **18** and **21** could be better. This is also probably the case for **19**, in which the methyl salicylate group has been interconverted.

In conclusion, the results of this work support (i) the essential role played by the hydroquinone moiety for tyrosine kinase inhibitory activity in the series of 5-[(2,5-dihydroxybenzyl)amino]salicylates; (ii) the importance of the reduced (CH_2NH) linking chain between the two aromatic rings, which allows the correct positioning of the 2,5-dihydroxybenzyl rings, possibly in a cis-like conformational arrangement, since derivatives of the trans imino series are inactive; (iii) the need to find additional favorable interactions with key residues at the active site, in order to increase biological activity, as compounds bearing salicylates and with good activity in vitro did not reach their target in cell cultures. This work is currently underway.

Experimental Section

Chemistry. Materials and Methods. All starting materials were purchased from Aldrich and Janssen. ^{1}H NMR spectra were recorded on a Bruker 270-MHz spectrometer. Chemical shifts are given in ppm relative to HMDS as internal standard. Signal multiplicity was designated according to the following abbreviations: s = singlet, d = doublet, t = triplet, q = quadrulet, m = multiplet, bs = broad signal. Melting points, determined on a electrothermal apparatus, are uncorrected. Column chromatography was performed on silica gel 60 (70–230-mesh ASTM) and TLC analysis on silica gel 60 F254 precoated plates. Elemental analyses for all imino and amino compounds were within ±0.4% of the theoretical value.

General Procedure of Imino Compound Formation. 5-[*N*-[(2′-Hydroxyphenyl)methylidene]amino]benzoic Acid Methyl Ester (2). To a solution of methyl 5-aminobenzoate hydrochloride (200 mg, 0.98 mmol, 1 equiv) in 10 mL of methanol were added 2-hydroxybenzaldehyde (120 mg, 0.98 mmol, 1 equiv) and triethylamine (0.14 ml, 0.98 mmol, 1 equiv). The reaction mixture was stirred at 60 °C for 8 h. After cooling, the precipitate was collected, washed with methanol, and dried to give 277 mg (85%) of **2**: mp 117–118 °C; ^{1}H NMR (DMSO) δ 12.15 (1H, s, OH), 10.5 (1H, s, OH), 8.92 (1H, s, CH=N), 7.78 (1H, d, J = 3 Hz, H6), 7.65 (2H, m, H4, H6′), 7.35 (1H, m, H4′), 7.05 (1H, d, J = 8.5 Hz, H3),

6.94 (2H, m, H5′, H3′), 3.85 (3H, s, CH_3). Anal. ($C_{15}H_{13}NO_4$) C, H, N.

General Procedure of Reduction. 2-Hydroxy-5-[*N*-[(2′-hydroxyphenyl)methyl]amino]benzoic Acid Methyl Ester (14). A solution of **2** (100 mg, 0.37 mmol) in 25 mL of methanol was stirred with 10 mg of 10% Pd/C under 1 atm of hydrogen for 4 h. The catalyst was filtered off. The solvent was then evaporated. Recrystallization from methanol and ethyl acetate gave 65 mg (65%) of **14**: mp 145.5–147.5 °C; ^{1}H NMR (DMSO) δ 9.75 (1H, s, OH), 9.46 (1H, s, OH), 7.12 (1H, d, J = 8.5 Hz, H6′), 7.0 (1H, t, J = 8.5 Hz, H4′), 6.9 (1H, d, J = 3 Hz, H6), 6.85 (1H, dd, J = 3, 8.5 Hz, H4), 6.78 (1H, d, J = 8.5 Hz, H3), 6.7 (1H, d, J = 8.5 Hz, H3′), 6.66 (1H, t, J = 8.5 Hz, H5′), 5.7 (1H, d, J = 6 Hz, NH), 4.1 (2H, d, J = 6 Hz, CH_2), 3.82 (3H, s, CH_3). Anal. ($C_{15}H_{15}NO_4$) C, H, N.

Molecular Modeling. All calculations were performed on a VaxStation 3500 and visualized on an Evans & Sutherland PS390 graphics system. The selected compounds **6** (imino) and **18** (amino) were built with the help of the fragment library in the SYBYL 5.5 molecular modeling package (Tripos Associates). They were minimized in a first step, in the all extended conformation, in order to reduce bond stretching and bending stress inherent in the construction of the molecules. Torsions α_1, α_2, and α_3 were each defined by the following sets of atoms: C_6'–C_1'–C–N, C_1'–C–N–C_5, and C–N–C_5–C_6 (see Figure 1). The conformational space was explored using the systematic conformer generating routine of SYBYL-SEARCH- with 30° steps around bonds associated to torsions α_1 and α_3 in **6**, and all three torsions α_1, α_2, and α_3 in **18**. For **6**, torsion α_2 was fixed at either 0° or 180° during the conformational search. The energy of each conformer generated by SEARCH was calculated with the Tripos force field; the energy expression includes the stretching, bending, torsional, 1–4 van der Waals and van der Waals terms; the molecules were electrically neutral and no calculations of the electrostatic terms were performed. The conformations thus produced gave then the intrinsic steric flexibility of the molecules. With a van der Waals general factor of 0.90, a total of 143 conformations for **6**, and 560 for **18** result, including redundant mirror image structures. For each compound, a unique set of nonredundant conformations was then selected. For practical purposes, those nonredundant conformations of **18** about 3 kcal/mol above the minimum (48) and all 72 nonredundant conformations for **6** were energy minimized using the conjugate gradient method and an energy convergence criterium of 0.05 kcal/mol. The energy intervals spanned by the 48 conformations of **18** is 5.3 kcal/mol, and that spanned by the 72 conformations of **6** is 6.5 kcal/mol.

Biological Experiments. The compounds were tested for their enzymatic activity using ER 22 cells membrane as enzyme source and the tridecapeptide RRSrc (RRLIEDAEYAARG) as substrate, as previously described by Onada.[7] The cellular activity was measured by the inhibition of EGF-stimulated DNA synthesis of ER 22 cells (CCL 39 cells transfected with EGF receptor) following the incorporation of methyl[^{3}H]thymidine.[28]

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