Structure-Activity Relationships in a Series of 5-[(2,5-Dihydroxybenzyl)amino]salicylate Inhibitors of EGF-Receptor-Associated Tyrosine Kinase: Importance of Additional Hydrophobic Aromatic Interactions

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Potent inhibitors of EGF-dependent protein tyrosine kinase (PTK) activity were synthesized in a series of 5-[(2,5-dihydroxybenzyl)amino]salicylates. Several of these compounds inhibited EGF-dependent DNA synthesis in ER 22 cells with $IC_{50} < 1$ μM. In this series of PTK inhibitors, the role of the salicylate moiety as a potential divalent ion chelator was tested and found to be nonessential in all cases. The length and ramification of the substituting carboxyl group were investigated to improve cellular bioavailability, and this analysis provided compounds with increased inhibitory effect on EGF-induced DNA synthesis. Salicylates esterified with long hydrophobic chains were shown to be noncompetitive inhibitors of ATP, in contrast to the free acid and methyl salicylate. Moreover, all the tested inhibitors were shown to be noncompetitive inhibitors of the peptide substrate. Structure-activity relationships allowed us to suspect a hydrophobic pocket in the tyrosine kinase domain, preferentially interacting with aromatic rings. Finally, the selectivity of the best inhibitors was tested against other kinases, and they were found to be selective for tyrosine kinase. They were also shown to be good inhibitors of EGF-receptor autophosphorylation.

Introduction

The involvement of the protein tyrosine kinase (PTK) activity of growth factor receptors in human tumor development, associated with a poor clinical diagnosis, is now well documented.[1,2] For example, the expression of the human epidermal growth factor receptor (EGFR) and its oncogenic analog human erb B_2 receptor (HER_2)/neu is greatly amplified in several human tumors,[3,4] accompanied by an overphosphorylation of their protein targets. This increased phosphorylation of substrate tyrosine residues by oncogenic PTK proteins is an essential step in the neoplastic transformation.[5,6] Site-directed mutagenesis experiments and the use of specific antibodies have also shown the requirement of an effective autophosphorylation process of tyrosine kinases for the subsequent phosphorylation of protein substrates and tumor development.[7-9]

Accordingly, specific inhibitors of protein tyrosine kinases can be useful in investigating the mechanisms of carcinogenesis, cell proliferation, and differentiation and could be effective in prevention and chemotherapy of cancer. For these reasons, numerous PTK inhibitors have already been developed (for review, see refs 10 and 11).

Rational approaches to design selective inhibitors of PTK activity associated with oncogene expression are multiple but difficult for several reasons: (i) the lack of precise structural data concerning the active site of the enzyme, (ii) the complexity of the enzymatic phosphoryl-transfer reaction which involves several steps, (iii) the multiplicity of endogenous substrates shared among the PTK family, and (iv) the large number of related protein kinases involved in normal growth and differentiation.

Design of PTK Inhibitors. Several natural products with different chemical structures, such as flavonoids,[12,13] erbstatin derivatives,[14,15] lavendustins,[16,17] the diuretic amiloride,[18] and the alcaloid staurosporine,[19] inhibit PTK activity and possess antiproliferative cellular activity.[20,21] These compounds are generally competitive inhibitors with ATP binding, and for this reason, it was suspected that they would not be sufficiently selective and thus be endowed with toxicity for nontumor cells.

Many synthetic PTK inhibitors have also been designed by taking into account the structure of tyrosine, such as the tyrphostins,[22,23] cinnamamides,[24,25] styrene derivatives,[26] or phenylhydrazones.[27] Among these compounds, the tyrphostins have been the most intensively studied. They were shown to be effective blockers of EGF-dependent cell proliferation[28] and to have antiproliferative activity in nude mice inoculated with a human squamous cancer, but only if the animals were treated during tumor growth and not after the tumor was developed.[29] Several tyrphostins were also reported (i) to be competitive inhibitors of substrate binding and noncompetitive inhibitors of adenosine triphosphate (ATP) binding,[30] (ii) to be selective for tyrosine kinases as opposed to serine or threonine kinases,[22,23,30] and (iii) to be able to discriminate in vitro between PTK activity associated with the EGF-receptor or other receptors.[31] Furthermore, tyrphostins were also reported to block sea star oocyte maturation, possibly by acting as Ser/Thr kinase inhibitors.[32]

In order to obtain inhibitors with increased affinity and specificity for the EGF-receptor, multisubstrate blocking agents were designed, based on a postulated structure of the transition state corresponding to phosphate transfer. In the active-site-proposal model, the substrate and ATP are located in hydrophobic sites, the γ atom of ATP is pentacoordinated, and the two β and γ phosphates form a complex with divalent metal ions.[33] Thus, dimeric molecules containing an adenyl base as the ATP moiety linked to an analog of tyrosine as a substrate mimic, with

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spacers of different lengths, have been designed, but these compounds displayed only moderate activity.[34-36] More recently, Traxler et al.[37] have postulated that (sulfonylbenzoyl)nitrostyrenes might inhibit the transition state of the PTK-induced transfer by binding to the substrate site, the nitrostyrene occupying the tyrosine site and the sulfonylbenzoyl moiety mimicking the diphosphate moiety. These compounds are relatively potent since they showed cellular antiproliferative activity at doses as low as 2 μM. The potency of these compounds was recently improved by substituting the sulfonylbenzoyl moiety with adenine 5'-substituted glutamates.[38]

In this work, we have selected the 5-[(2,5-dihydroxybenzyl)amino]salicylate moiety as a simplified model of lavendustin A, one of the most potent in vitro PTK inhibitors.[16] We had previously confirmed that the third aromatic ring of lavendustin A is not essential for PTK activity.[16,39] We had also shown that the two hydroxyl groups of the hydroquinone ring are absolutely necessary and that the salicylate moiety might be important for recognition of the ATP-binding site.[39] Chemical modifications are now reported to investigate the role of the salicylate in more depth. Addition of a CH_2 group between the carboxylate and the phenyl ring of the salicylate should decrease the metal chelating ability of the inhibitors, while replacing the carboxylate by a hydroxamate group might preserve or increase this ability. The results are discussed in relation with the CO-group electron-donor potency of the different compounds.

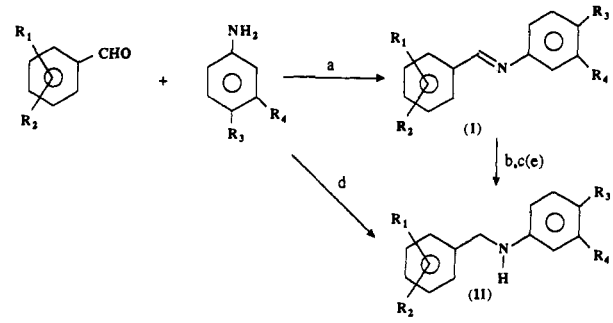
Otherwise, chemical modifications of the inhibitors were tried to increase their cellular penetration. Intermediate compounds protected by hydrophobic residues were found to be active at relatively low doses. Therefore, in order to test whether these compounds were interacting with a hydrophobic subsite in the kinase domain, several alkyl and phenylalkyl salicylates of varying size were synthesized and their PTK inhibitory potency evaluated. These molecules demonstrated that a hydrophobic pocket might be present in the kinase domain and that this pocket could bind hydrophobic substituents with aromatic rings whose location and size are well defined.

Chemistry

The compounds containing a rigid imino spacer (series I) or their flexible amino counterparts (series II) were prepared following the general procedure described in Scheme 1. Thus, the condensation of 2-formylhydroquinone with the adequately substituted anilines provided imino compounds which were isolated, or not, before their catalytic hydrogenation or chemical reduction to provide 5-[(2,5-dihydroxybenzyl)amino]salicylates or -phenylacetates. Relatively few compounds of the chemical intermediates, the nitro or aniline precursors, had already been described;[40-42] thus, their syntheses and characteristics are reported in Schemes 2 and 3 and Tables 1 and 2, respectively.

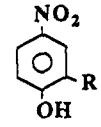
Derivatives of the aminophenylacetic series were obtained by nitration of 2-hydroxyphenylacetic acid and isolation of the 5-nitro isomer followed by esterification and catalytic hydrogenation. In order to obtain hydroxamate precursors in this series, 5-nitro-2-hydroxyphenylacetic acid (A_1) was condensed with the protected hydroxylamines and subsequently reduced catalytically or chemically. In the 5-aminosalicylic series, hydroxamates were obtained either from the 5-nitrosalicylic acid by condensation with the *O*-substituted hydroxylamine and further catalytic reduction or by protecting the amino group of the commercial 5-aminosalicylic acid by Boc_2O (Boc, (*tert*-butyloxy)carbonyl) before coupling with the *O*-substituted hydroxylamine and deprotecting the amino group by the action of trifluoroacetic acid (TFA). The amine obtained was then condensed with the formylhydroquinone. All the 5-aminosalicylates were obtained by esterification of the 5-nitrosalicylic acid using several

Scheme 1. General Synthesis of Imino (I) and Amino (II) Compounds[a]



[a] (a) Condensation by heating in methanol, DMF, or toluene; (b_1) catalytic hydrogenation with palladium in methanol, dichloromethane, or ethyl acetate. (b_2) catalytic hydrogenation with nickel in methanol; (b_3) reduction with $NaBH_4$ in methanol; (b_5) reduction with aqueous $Na_2S_2O_4$; (c_1) $SOCl_2$ and methanol or ethanol; (c_2) ROH/DCC/DMAP; (c_3) $SOCl_2$ in toluene, then ROLi in THF; (c_4) DCC in pyridine/DMF, then ROH in CH_2Cl_2 or AcOEt; (d_1) refluxed in methanol or toluene, then reduction with $NaBH_3CN$ in methanol or toluene; (d_2) refluxed in methanol or toluene, then catalytic hydrogenation with Pd in dichloromethane or ethyl acetate; (e) refluxed in 6 N aqueous HCl.

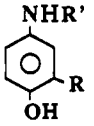
Table 1. Nitro Intermediates: Preparation and Properties



cmpd	R	method	yield (%)	mp (°C)
A_1	CH_2-CO_2H	f	43	159.5–160.5
A_2	CH_2-CO_2-Me	c_1	69	155–156.5
A_3	CH_2-CO_2-Et	c_1	85	154–155.5
A_4	CH_2-CO-NH-OtBu	g	83	182–184
A_5	CH_2-CO-NH-O-CH_2Ph	g	38	149–150
A_6	CH_2-PO_3-Et_3	h	100	139–140
A_9	CO-NH-O-Me	g	51.8	175–176.5
A_{10}	CO-NH-OtBu	g	50	184.5–185.5
A_{15}	CO_2-tBu	c_2	82	81–81.5
A_{16}	CO_2-$(CH_2)_2$-tBu	c_2	84	54–55
A_{17}	CO_2-CH_2-CH(Me)-CH_2-tBu	c_2	85	51–52
A_{18}	CO_2-$(CH_2)_2$-CH(Me)-CH_2-tBu	c_2	85	viscous oil
A_{19}	CO_2-$(CH_2)_2$-CH(Me)-$(CH_2)_3$-iPr	c_2	90	viscous oil
A_{20}	CO_2-$(CH_2)_{15}$-CH_3	c_3	48	viscous oil
A_{21}	CO_2-CH_2-cC_6H_{11}	c_2	71	viscous oil
A_{22}	CO_2-$(CH_2)_2$-Ada	c_3	61	106
A_{23}	CO_2-Ph	c_4	49	152[a]
A_{24}	CO_2-CH_2-Ph	c_1	68	85[b]
A_{25}	CO_2-$(CH_2)_2$-Ph	c_2	86	111.5–112
A_{26}	CO_2-$(CH_2)_3$-Ph	c_2	88	viscous oil
A_{27}	CO_2-$(CH_2)_4$-Ph	c_2	81	viscous oil
A_{28}	CO_2-CH_2-CH(Me)-CH_2-Ph	c_2	89	64–66
A_{29}	CO_2-$(CH_2)_2$-CH(Me)-Ph	c_2	72	oil
A_{30}	CO_2-CH_2-CH=CH-Ph	c_4	93	89
A_{31}	CO_2-CH_2-(iPr)Ph	c_2	82	174–175
A_{32}	CO_2-CH_2-(3,5-diMe)Ph	c_2	76	70–71
A_{33}	CO_2-(3-OH)Ph	c_4	47	156
A_{34}	CO_2-(4-Ph)Ph	c_4	78	102
A_{35}	CO_2-1-naphthyl	c_4	58	152
A_{36}	CO_2-2-naphthyl	c_4	38	168

[a] 148–150 °C in ref 41. [b] 83.5 °C in ref 42.

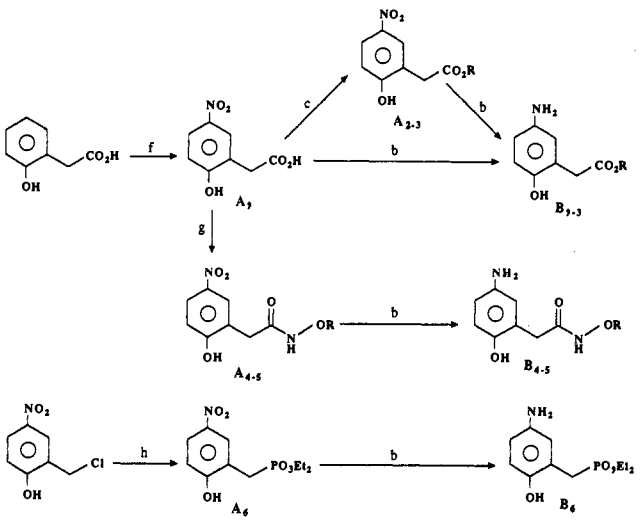
Table 2. Amino Intermediates: Preparations and Properties



cmpd	R'	R	method	yield (%)	mp (°C)
B_1	H	CH_2-CO_2H	b_1	66	218–218.5 (HCl)[a]
B_2	H	CH_2-CO_2-Me	b_1	98	125–126.5
B_3	H	CH_2-CO_2-Et	b_1	98	102–104
B_4	H	CH_2-CO-NH-OtBu	b_1	96	140–142
B_5	H	CH_2-CO-NH-OCH_2Ph	b_5	73	150–151[a]
B_6	H	CH_2-PO_3-Et_2	b_1	94	118–120
B_9	H	CO-NH-OMe	b_1	80	189–190
B_{10}	H	CO-NH-OtBu	b_1	100	146–147
B_{11}	H	CO-NH-OCH_2-Ph	b_5	90	200–201 (CF_3CO_2H)[b]
B_{15}	H	CO_2-tBu	b_1	100	63–64.5
B_{16}	H	CO_2-$(CH_2)_2$-tBu	b_1	100	viscous oil
B_{17}	H	CO_2-CH_2-CH(Me)-CH_2-tBu	b_1	97	44–45.5
B_{18}	H	CO_2-$(CH_2)_2$-CH(Me)-CH_2-tBu	b_1	100	viscous oil
B_{19}	H	CO_2-$(CH_2)_2$-CH(Me)-$(CH_2)_3$-iPr	b_1	98	viscous oil
B_{20}	H	CO_2-$(CH_2)_{15}$-CH_3	b_1	85	viscous oil
B_{21}	H	CO_2-CH_2-cC_6H_{11}	b_1	92	78–79
B_{22}	H	CO_2-$(CH_2)_2$-Ada	b_1	92	109
B_{23}	H	CO_2-Ph	b_1	88	162*
B_{24}	H	CO_2-CH_2-Ph	b_4	48	188 (HCl)[a]*
B_{25}	H	CO_2-$(CH_2)_2$-Ph	b_1	97	76–76.5
B_{26}	H	CO_2-$(CH_2)_3$-Ph	b_1	94	viscous oil
B_{27}	H	CO_2-$(CH_2)_4$-Ph	b_1	90	viscous oil
B_{28}	H	CO_2-(CH_2)-CH(Me)-CH_2-Ph	b_1	50	viscous oil
B_{29}	H	CO_2-$(CH_2)_2$-CH(Me)-Ph	b_1	50	viscous oil
B_{30}	H	CO_2-CH_2-CH=CH-Ph	b_4	94	141
B_{31}	H	CO_2-CH_2-(4-iPr)Ph	b_1	39	oil
B_{32}	H	CO_2-CH_2-(3,5-diMe)Ph	b_1	49	95
B_{33}	H	CO_2-(3-OH)Ph	b_1	89	128
B_{34}	H	CO_2-(4-Ph)Ph	b_1	89	173
B_{35}	H	CO_2-1-naphthyl	b_1	85	130
B_{36}	H	CO_2-2-naphthyl	b_1	78	143
C_{12}	Boc	COOH	i	97	278 (dec)
C_{11}	Boc	CO-NH-OCH_2-Ph	g	73	121.5–123.5

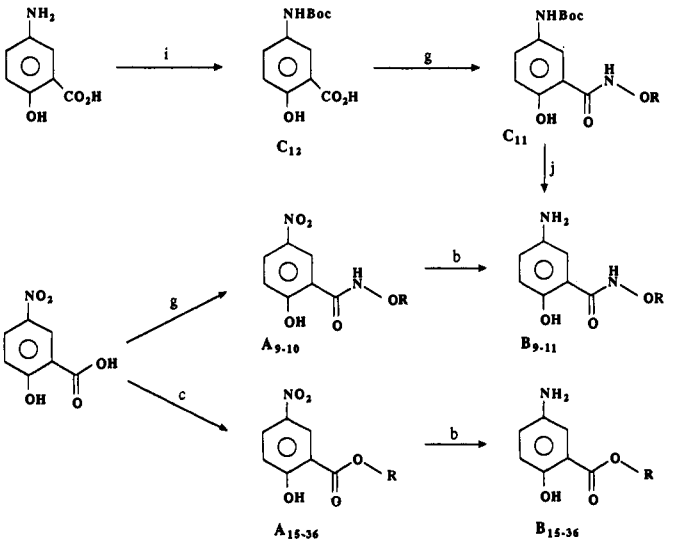
[a] As chlorohydrate salt. [b] As trifluoroacetate salt; dec is decomposition; * = melting points are not given in literature.

Scheme 2. Phenylacetic Series Intermediates[a]



[a] (b_1) Catalytic hydrogenation with palladium in methanol, dichloromethane, or ethyl acetate; (b_2) catalytic hydrogenation with nickel in methanol; (b_3) reduction with $NaBH_4$ in methanol; (b_5) reduction with aqueous $Na_2S_2O_4$; (c_1) $SOCl_2$ and methanol or ethanol; (c_2) ROH/DCC/DMAP; (c_3) $SOCl_2$ in toluene, then ROLi in THF; (c_4) DCC in pyridine/DMF, then ROH in CH_2Cl_2 or AcOEt; (f) HNO_3 in H_2SO_4; (g) NH_2OR, HCl, and Et_3N in $CHCl_3$ and THF; (h) $P(OEt)_3$.

Scheme 3. Benzoic Series Intermediates[a]

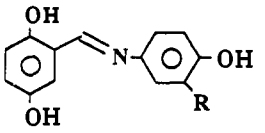


[a] (b_1) Catalytic hydrogenation with palladium in methanol, dichloromethane, or ethyl acetate; (b_2) catalytic hydrogenation with nickel in methanol; (b_3) reduction with $NaBH_4$ in methanol; (b_5) reduction with aqueous $Na_2S_2O_4$; (c_1) $SOCl_2$ and methanol or ethanol; (c_2) ROH/DCC/DMAP; (c_3) $SOCl_2$ in toluene, then ROLi in THF; (c_4) DCC in pyridine/DMF, then ROH in CH_2Cl_2 or AcOEt; (g) NH_2OR, HCl, and Et_3N in $CHCl_3$ and THF; (i) Boc_2O in dioxane; (j) TFA in $CHCl_3$.

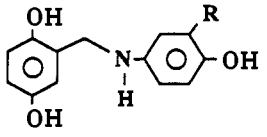
different methods (see Schemes 2 and 3) followed by reduction of the nitro group, condensation with the formylhydroquinone, and reduction of the imino group. Only methyl and ethyl 5-[(2,5-dihydroxybenzyl)amino]-salicylates could be obtained by direct esterification of the 5-[(2,5-dihydroxybenzyl)amino]salicylic acid precursor.

Table 3. Preparations and PTK Inhibitory Activity in the Imino and Amino Series[a]

Imino series



Amino series



R	cmpd	method	vitro IC_{50} (μM)	cell IC_{50} (μM)	cmpd	method	vitro IC_{50} (μM)	cell IC_{50} (μM)
CH_2-CO_2H	I_1	a	35	60	II_1	b_1	6	38
CH_2-CO_2-Me	I_2	a	100	20	II_2	b_2	3	12
CH_2-CO_2-Et	I_3	a	100	20	II_3	b_2	7	11
CH_2-CO-NH-OtBu	I_4	a	31	30	II_4	b_1	5	46
CH_2-CO-NH-OCH_2-Ph	I_5	a	50	61% at 10	II_5	b_3	1	16
CH_2-PO_3-Et_2	I_6	a	$\gg$100	75	II_6	b_1	4	70
CH_2-PO_3-H_2	I_7	n.p.			II_7	e	3	>100
CO-NH-OH	I_8	n.p.			II_8	b_1	0.4	40
CO-NH-OMe	I_9	a	27	$\gg$100	II_9	b_1	0.3	20
CO-NH-OtBu	I_{10}	a	20% at 10	$\gg$20*	II_{10}	b_1	0.1	35% at 20
CO-NH-OCH_2-Ph	I_{11}	a	8% at 10	43% at 5	II_{11}	b_3	0.05	41% at 10
CO-OH	I_{12}**	a	5	100	II_{12}**	b_1	0.03	92
CO_2-Me	I_{13}**	a	56% at 100	n.t.	II_{13}**	b_1	0.6	9
CO_2-Et	I_{14}	a	50	40	II_{14}	c	0.4	6.1
CO_2-tBu	I_{15}	a	15% at 10	$\gg$10	II_{15}	b_2	1	8
CO_2-$(CH_2)_2$-tBu	I_{16}	a	10% at 10	$\gg$2*	II_{16}	b_2	0.7	7
CO_2-CH_2-CH(Me)-CH_2-tBu	I_{17}	a	$\gg$10*	10	II_{17}	b_2	~4	0.8
CO_2-$(CH_2)_2$CH(Me)-CH_2-tBu	I_{18}	a	20% at 10	42% at 5	II_{18}	b_2	$\gg$1*	0.8
CO_2-$(CH_2)_2$-CH(Me)-$(CH_2)_3$-iPr	I_{19}	a	20% at 10	5	II_{19}	b_2	7	0.8
CO_2-$(CH_2)_{15}$-CH_3	I_{20}	n.i.			II_{20}	d_1	$\gg$5*	$\gg$5*
CO_2-CH_2-cC_6H_{11}	I_{21}	a	n.t.	n.t.	II_{21}	b_2	1.2	2.5
CO_2-$(CH_2)_2$-Ada	I_{22}	n.i.			II_{22}	d_1	$\gg$5*	1.25
CO_2-Ph	I_{23}	n.i.			II_{23}	d_1	0.07	10
CO_2-CH_2-Ph	I_{24}	n.i.			II_{24}	d_1	0.08	2.5
CO_2-$(CH_2)_2$-Ph	I_{25}	a	n.t.	n.t.	II_{25}	b_2	0.13	3
CO_2-$(CH_2)_3$-Ph	I_{26}	a	n.t.	n.t.	II_{26}	b_2	0.03	1.5
CO_2-$(CH_2)_4$-Ph	I_{27}	a	n.t.	n.t.	II_{27}	b_2	0.4	1.25
CO_2-CH_2-CH(Me)-CH_2-Ph	I_{28}	a	n.t.	n.t.	II_{28}	b_2	0.36	3.5
CO_2-$(CH_2)_2$-CH(Me)-Ph	I_{29}	a	n.t.	n.t.	II_{29}	b_2	0.4	2.7
(*E*) CO_2-CH_2-CH=CH-Ph	I_{30}	n.i.			II_{30}	d_1	1.3	2
CO_2-CH_2-(4-iPr)Ph	I_{31}	a	n.t.	n.t.	II_{31}	b_2	1	1.4
CO_2-CH_2-(3,5-diMe)Ph	I_{32}	a	n.t.	n.t.	II_{32}	b_2	3	1.6
CO_2-(3-OH)Ph	I_{33}	n.i.			II_{33}	d_2	0.09	$\gg$5*
CO_2-(4-Ph)Ph	I_{34}	n.i.			II_{34}	d_2	5	42% at 5
CO_2-1-naphthyl	I_{35}	n.i.			II_{35}	d_2	0.11	4.4
CO_2-2-naphthyl	I_{36}	n.i.			II_{36}	d_2	0.09	21% at 5

[a] The inhibitory potency of the various compounds against tyrosine kinase activity associated with EGFR was evaluated using ER 22 cell membranes as an enzyme source and the tridecapeptide RR-Src (RRLIEDAEYAARG) as the phosphoryl-acceptor substrate as described by Onada.[16] The inhibitor activity of the compounds on EGF-stimulated DNA synthesis was assessed by measuring [^{3}H]Me-dT incorporation into ER 22 cells as described by L'Allemain.[43] * = due to their weak water solubility, inhibitory potency of these compounds could not be tested at higher doses; their inhibitory effect is 0% at these doses. ** = compounds already described in ref 39. n.i., not isolated; n.t., not tested; n.p., not prepared.

Results and Discussion

Structure–Activity Relationships. The inhibitory potency of the various compounds against protein tyrosine kinase activity associated with EGFR was evaluated using ER 22 cell membranes[43] as an enzyme source and the tridecapeptide RR-Src (RRLIEDAEYAARG)[16] as the phosphoryl acceptor substrate. The inhibitory activity of the compounds on EGF-stimulated DNA synthesis at the cellular level was assessed by measuring [^{3}H]Me-dT incorporation into ER 22 cells.

The results, reported in Table 3, show that compounds of the imino series are much less active than compounds of the amino series. As discussed in a preceding paper, this difference might be due to conformational preferences.[39] For the amino series, energetical calculations showed two families of nonplanar pseudo-cis and -trans arrangements around the C–N bond with similar low energies, demonstrating the possible existence of several conformer arrangements in solution. However, only one trans arrangement was observed in the weakly active series of rigid imino compounds, suggesting that inhibitors of the amino series might adopt a bioactive conformation which resembles a cis arrangement.

In the series of amino compounds, some inhibitors (II_{11}, II_{12}, II_{23}, II_{24}, II_{26}, II_{33}, and II_{36}) exhibited in vitro activity comparable with that of the best PTK inhibitors already reported in the literature, such as the lavendustins[16,17] or quinazolines.[44] As already observed for most other PTK inhibitors, the compounds presented in this paper were almost 2 orders of magnitude more potent in vitro than in the cell-based assay.[16] This could be due to the fact that, for compounds which behave as competitive inhibitors of ATP, their inhibitory potency is reduced by the high intracellular concentration of ATP and/or because they have poor cellular penetration, due to their high polarity.

In an attempt to improve the cell penetration of the inhibitors, compounds with different hydrophobic chains lengths were prepared. Some of these had IC_{50} values < 1 μM in the EGF-dependent DNA assay on ER 22 cells (Table 3). Thus, the biological activity of compounds II_{17}–II_{19} showed that the optimal length of the hydrophobic

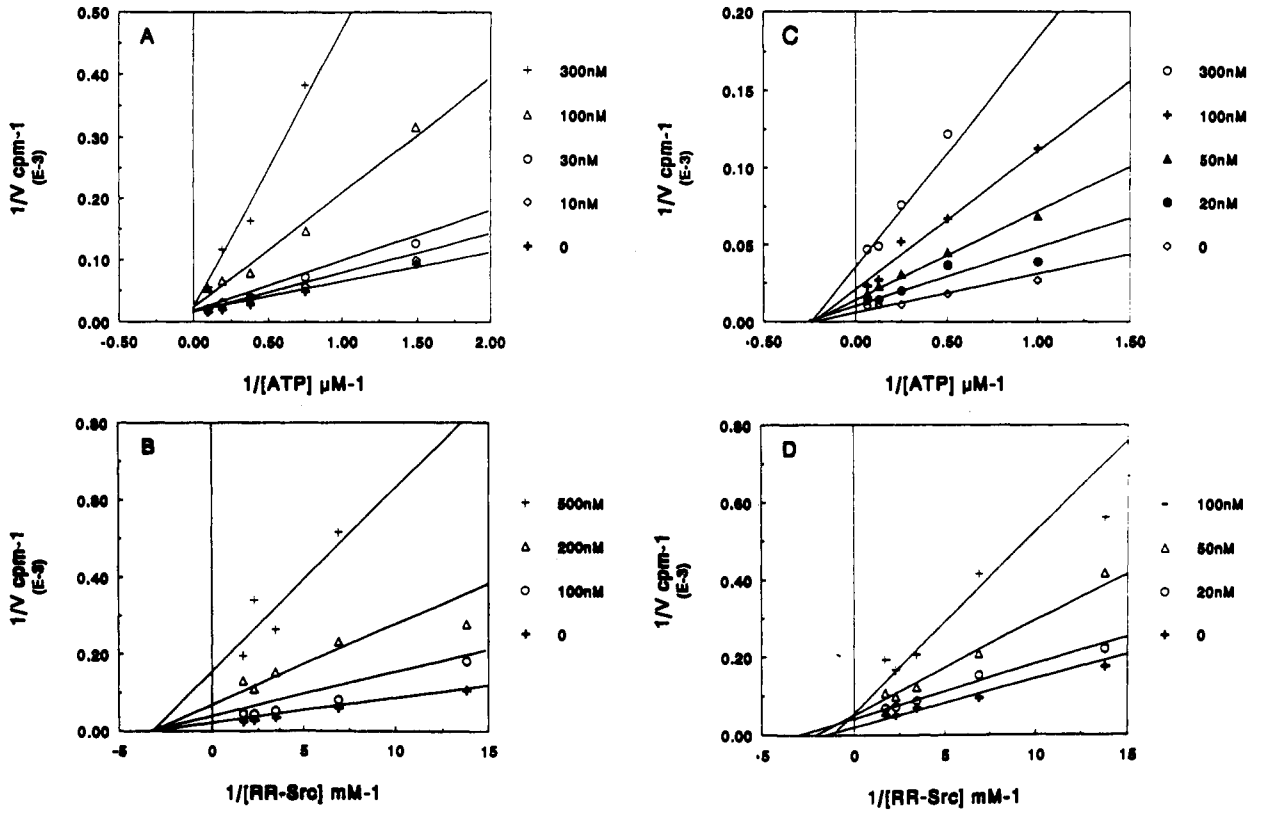


Figure 1. Lineweaver–Burk plot of the EGF-receptor tyrosine kinase assay with RR-Src as substrate, performed as described in the Experimental Section. The inhibitors were incubated at the indicated concentrations, in the presence of a fixed RR-Src concentration and various ATP concentrations for kinetic analysis versus ATP and in the presence of a fixed ATP and various RR-Src concentrations for kinetic analysis versus RR-Src. Double-reciprocal plots are shown: (A) II_{12} versus ATP, (B) II_{12} versus RR-Src, (C) II_{26} versus ATP, and (D) II_{26} versus RR-Src.

chain is 5–8 carbons with methyl substituents as ramifications. Longer and linear chains are less favorable, as shown by the weaker activity of compounds II_{20}–II_{22}.

Among compounds exhibiting the best activities in the cell-based assay, it is intriguing to note that compounds such as II_{17}–II_{19} were more efficient in inhibiting the DNA synthesis in EGFR-dependent cells than in inhibiting the EGFR-mediated phosphorylation of the peptide substrate. Similar results have already been observed with the tyrphostin[31] and thiazolidinedione PTK inhibitors.[45] It has been suggested that a cellular accumulation of the inhibitors, which are very hydrophobic, or their inhibitory action on unknown PTKs downstream in the EGF-signaling pathway might account for this observation.[31,45] Either of these explanations could be valid for our compounds, but they could also interact with another EGF-dependent target in the cells.

It is interesting to note that when the length of the hydrophobic chain increases (compounds II_{13}–II_{19}), the inhibitory effect, measured in vitro on the kinase, decreases. This decrease in inhibitory potency might be due to decreased Mg^{2+} chelation power of the substituted salicylates as compared with the free salicylic moiety in II_{12}.

Investigation of the Salicylate Interaction with Mg^{2+}. A decrease of the Mg^{2+} chelating power of alkyl salicylates, as compared to the free acid II_{12}, appears to be supported by the lower inhibitory potency of compound II_1 as compared to II_{12}, since in II_1 the COOH and OH groups of the salicylate are separated by a methylene group. Hydroxamate derivatives, which are known to be good metal chelators, were also synthesized. The hydroxamate II_8 was found to be a potent inhibitor of the kinase in vitro, and its activity could also be explained by its magnesium chelating ability. Thus, the ability for chelating Mg^{2+} seems important for certain inhibitors. Nevertheless, intermediate compounds II_{10} and II_{11}, protected with hydrophobic substituents, showed improved inhibitory potencies with a preference for an aromatic ring (II_{11} is better than II_{10}), suggesting that these hydrophobic substituents might also have additional favorable interactions within the kinase domain, compensating for their magnesium chelating potency decrease.

Investigation of Aromatic Salicylate Substitution Inhibitor Potency. On the basis of the observation that an aromatic ring might increase inhibitory potency and in order to preserve cellular penetration, esters with hydrophobic chains bearing different aromatic chains were prepared. The presence of an aromatic ring at the end of the linker enhanced the potency of the inhibitors (compounds II_{23}–II_{27}), and the linker length was optimized in compound II_{26}, which had good inhibitory activity for the kinase (IC_{50} = 30 nM).

As observed with the alkyl esters, substitution by methyl groups on the chain bearing an aromatic ring (compounds II_{28} and II_{29}) is not advantageous for in vitro activity. The rigidification of the linker, as shown by comparing II_{26} and II_{30}, and the hydrogenation of the aromatic ring to a cyclohexyl ring, as shown by a comparison of II_{21} and II_{24}, reduced activity. Neither esterification by larger aromatic rings, such as α or β naphthyl groups (compounds II_{35} and II_{36} as compared with II_{23}), or biphenyl rings (compound II_{34}) nor substitution of the phenyl ring by hydrophobic (II_{31} and II_{32} as compared with II_{24}) or hydrophilic

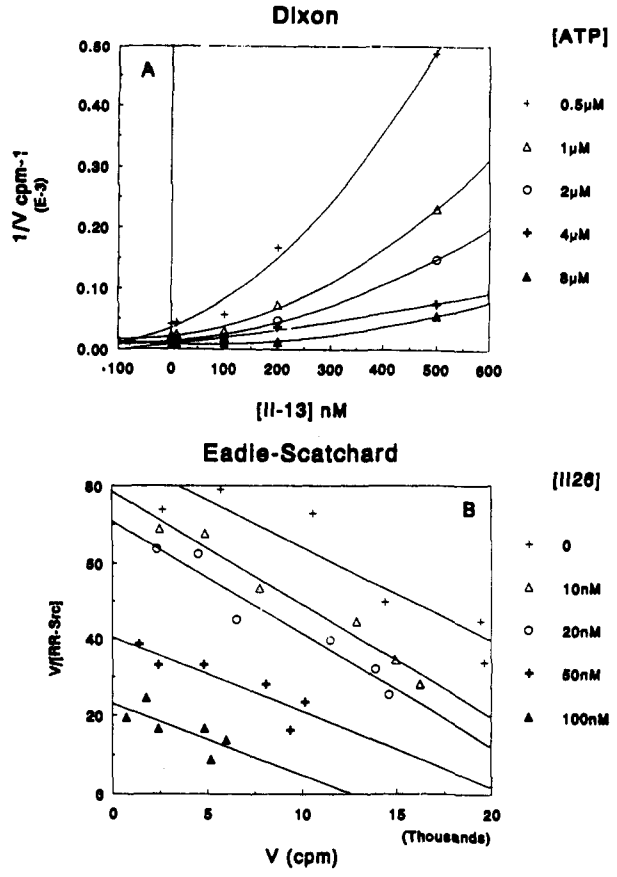


Figure 2. (A) Competition analysis of II_{13} versus ATP using a Dixon-plot representation showing a parabolic competitive inhibition mode. (B) Competition analysis of II_{26} versus RR-Src using Eadie–Scatchard representation showing a noncompetitive inhibition mode.

substituents (II_{33}) produced better compounds. Taken together, these results suggest that the lipophilic esters and hydroxamates with chains containing aromatic rings might have favorable interactions within a hydrophobic site in the kinase domain, whose size is optimally adapted for the phenylpropyl chain of compounds II_{26}. Moreover, the majority of these esters bearing aromatic rings are relatively good inhibitors of EGF-dependent DNA synthesis, with an optimization for the chain of compounds II_{26}.

Mode of Inhibition. Kinetic Analyses. In order to understand the inhibition mode of the inhibitors, enzymatic kinetic studies were performed. The salicylate compound II_{12} (IC_{50} in vitro = 30 nM; IC_{50} cells = 92 μM) was found to be competitive versus ATP and noncompetitive versus RR-Src in Lineweaver–Burk- (Figure 1A,B) and Dixon-plot representations (data not shown). This competitive inhibition of ATP binding is in agreement with the discussion in the Structure–Activity Relationships section, assuming an essential role of the salicylate group in chelating Mg^{2+} in the complex formed with ATP β and γ phosphates. This hypothesis was confirmed by kinetic analysis of methyl ester II_{13}. Using a Lineweaver–Burk representation, we had previously shown that this compound was a competitive inhibitor of ATP and a noncompetitive inhibitor of RR-Src[39] with a reduced IC_{50} in vitro (0.6 μM) as compared to that of II_{12} (30 nM). Using a Dixon-plot representation (Figure 2A), we obtained parabolic curves for inhibition of ATP binding, showing that compound II_{13} is a slow parabolic competitive inhibitor of ATP.[46] This suggests that two molecules of

Table 4. Inhibitory Potency of Selected Compounds against Different Kinases Activity and EGFR Autophosphorylation[a]

	vitro				cell
	RR-Src IC_{50} (μM)	EGFR autophosph IC_{50} (μM)	PKC IC_{50} (μM)	PKA IC_{50} (μM)	ER 22 [^{3}H]dT inc IC_{50} (μM)
II_{12}	0.03	0.15	>100	>100	92
II_{13}	0.6	1	400	150	9
II_{17}	4	8	300	10	0.8
II_{19}	7	25	20% at 10	10	0.8
II_{25}	0.13	0.20	42% at 30	4	3
II_{26}	0.03	0.7	>20	11	1.5

[a] Inhibitory potency against EGFR tyrosine kinase activity using the peptide RR-Src as substrate, inhibitory potency on the EGFR autophosphorylation, and inhibitory potency against PKC and PKA were measured in vitro, and inhibitory potency against EGF-dependent DNA synthesis was measured on ER 22 cells for several selected compounds (see the Experimental Section).

II_{13} might bind to the ATP-binding site, probably due to a decrease in the interaction of the methyl ester carboxyl group with Mg^{2+} in the ATP-binding site.

II_{26} is among the best compounds of this series. The presence of an aromatic hydrophobic ester chain in II_{26} gives a good inhibitory effect on the kinase activity (IC_{50} = 30 nM) and on EGF-stimulated DNA synthesis (IC_{50} = 1.5 μM). Kinetic analysis with compound II_{26} showed a noncompetitive inhibition of ATP in Lineweaver–Burk (Figure 1C) and Dixon (data not shown) representations. These results suggest that this compound does not interact by its salicyl group with Mg^{2+} within the ATP-binding site, probably due to steric hindrance. Kinetic analysis of the inhibition of RR-Src binding led to unusual plots using both double-reciprocal Lineweaver–Burk (Figure 1D) and Dixon (data not shown) representations. In these graphical representations, the curves neither intercepted at one common point nor were they parallel. In contrast, parallel curves (Figure 2B), obtained using Eadie–Scatchard analysis, suggested that II_{26} is a noncompetitive inhibitor of RR-Src.[46] These results suggest that compound II_{26} binds to the kinase receptor at a site which is distinct from the binding sites for ATP and the peptide substrate and that it induces a conformational change which reduces the binding affinities for the two substrates.

In summary, it can be concluded from the kinetic analyses that compounds II_{12} and II_{13} have a direct or partial interaction within the ATP-binding site, respectively. The interaction with II_{13}[47,48] suggests an extented catalytic center in the ATP-binding site. It seems that compounds with aromatic hydrophobic side chains like II_{26} may bind to a different site in the receptor kinase domain and perhaps to an allosteric site, thus forming different enzyme–inhibitor complexes. The lack of a detailed understanding of the three-dimensional structure of a PTK catalytic domain and the observation of different mechanisms of inhibition increase the challenge for the development of selective synthetic PTK inhibitors.

Receptor Autophosphorylation. Since receptor autophosphorylation, triggered by growth factor binding, is considered to be the first step in signal transduction and a prerequisite for further phosphorylation of the substrate proteins,[49] the inhibitors were tested for their effect on this process in vitro.

As shown in Table 4, inhibitors of RR-Src phosphorylation also block the EGFR autophosphorylation in vitro. However, as already observed in several series,[16,31] the doses needed to inhibit receptor autophosphorylation are higher

than those needed to inhibit peptide phosphorylation. Each of the compounds II_{13}, II_{17}, and II_{25}, is equipotent on both processes. On the other hand, compounds II_{12} (competitive with ATP) and II_{26} (noncompetitive with ATP), the best in vitro inhibitors, are much less effective in inhibiting EGF-receptor autophosphorylation than in inhibiting substrate phosphorylation. Thus, no correlation can be made with the enzymatic activity in vitro or with the site of action of the inhibitors. Moreover, it is not known whether partial or total inhibition of the EGFR autophosphorylation process is needed to inhibit substrate phosphorylation.

Selectivity Characteristics. The selectivity of the most interesting tyrosine kinase inhibitors was evaluated in vitro versus the Ser/Thr kinase PKC and the cyclic-AMP-dependent PKA. Compounds II_{12}, II_{13}, II_{25}, and II_{26} are 10^2–10^5 times more potent in inhibiting PTKs than PKC or PKA. Therefore, the low potency of II_{12} and II_{13} in the cellular model is probably due to their poor cellular penetration. Compounds II_{25} and II_{26}, which have a more favorable hydrophobic–hydrophilic balance, have significantly improved inhibitory effects on EGF-induced DNA synthesis. Compounds II_{17} and II_{19}, which have lower inhibitory potencies against PTK and have, in addition, less selectivity for PKA, are nevertheless potent inhibitors of EGF-induced DNA synthesis on cells. The fact that compound II_{26} is a noncompetitive inhibitor of ATP binding and has a good selectivity for PTK as compared to other kinases such as PKC and PKA, in addition to a good correlation for the inhibition of RR-Src phosphorylation and the EGF-induced DNA synthesis on ER 22 cells, makes II_{26} the best candidate, in this series, for inhibiting the protein tyrosine kinase activity associated with the EGF-receptor.

Conclusion

Potent inhibitors of EGF-receptor-associated protein tyrosine kinase activity, belonging to the 5-[(2,5-dihydroxybenzyl)amino]salicylate series, have been designed. Some of them act as competitive inhibitors of ATP and others as noncompetitive of ATP. All of the inhibitors tested were noncompetitive inhibitors of the peptide substrate RR-Src.

Structure–activity relationships have been performed, and the presence of a hydrophobic aromatic chain appears to greatly enhance inhibitory potency in the ester series. This is not surprising since 5-[(2,5-dihydroxybenzyl)-amino]salicylates are derived from lavendustin A, which contains three aromatic rings.[16] However, taken together with the kinetic data, these results suggest that derivatives like II_{26} target a hydrophobic subsite, within the kinase domain, distinct from the ATP- and substrate-binding sites. However, it is surprising that small modifications in the chemical structure of the inhibitors are able to completely modify their mode of PTK inhibition. These results suggest that the enzyme might be strongly conformationally modulated.

The inhibitors obtained in this study are also blockers of receptor autophosphorylation, and the majority are selective for tyrosine versus Ser/Thr kinases. Their potency in inhibiting the growth of tumors bearing amplified EGFR or HER_2 is under study. Further work is also underway to improve their efficacy, and molecular modeling of the receptor catalytic site,[50] recently reported, should help for the research of inhibitors with higher selectivity and affinity.

Experimental Section

Chemistry. Materials and Methods. All starting materials were purchased from Aldrich and Janssen. ^1H 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 Imine Formation: Method a. 2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methylidene]amino]phenylacetic Acid (I_1). To a solution of B_1 (200 mg, 0.98 mmol, 1 equiv) in 10 mL of methanol were added 2,5-dihydroxybenzaldehyde (136 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. The solvent was removed on a rotary evaporator. Purification by flash chromatography over silica gel (4:1, dichloromethane:methanol) gave 200 mg of I_1 (71%): mp >300 °C; ^1H NMR (DMSO) δ 12.32 (1H, s, OH), 9.05 (1H, s, OH), 8.7 (1H, s, CH=N), 7.1 (2H, m, H6, H4), 6.9 (1H, d, J = 3 Hz, H6′), 6.78 (1H, dd, J = 3 Hz, 8.5 Hz, H4′), 6.7 (1H, d, J = 8.5 Hz, H3′), 6.66 (1H, d, J = 8.5 Hz, H3), 3.35 (2H, s, CH_2). Anal. ($C_{15}H_{13}NO_5$) C, H, N.

General Procedures of Reduction. Catalytic Hydrogenation: Method b_1. 2-Hydroxy-5-aminophenylacetic Acid Methyl Ester (B_2). A solution of A_2 (300 mg, 1.92 mmol) in 20 mL of methanol was stirred with 30 mg of 10% Pd/C under 1 atm of hydrogen for 4 h. The catalyst was filtered off. The solvent was then stripped off on a rotary evaporator to provide 270 mg (98%) of B_2: mp 125–126.5 °C; ^1H NMR (DMSO) δ 8.38 (1H, s, OH), 6.45 (1H, d, J = 8.5 Hz, H3), 6.3 (1H, d, J = 3 Hz, H6), 6.28 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 4.45 (2H, s, NH_2), 3.52 (3H, s, CH_3), 3.38 (2H, s, CH_2).

Catalytic Hydrogenation: Method b_2. 2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methyl]amino]phenylacetic Acid Methyl Ester (II_2). A solution of I_2 (200 mg, 6.64 mmol) in 20 mL of methanol was stirred with 20 mg of Raney Ni under 1 atm of hydrogen for 10 h and then filtered and washed with methanol. The solvent was stripped off on a rotary evaporator. The residue was purified by flash chromatography over silica gel (9:1, dichloromethane:methanol) to provide 143 mg (71%) of II_2: mp 149–150 °C; ^1H NMR (DMSO) δ 8.65 (1H, s, OH), 8.45 (1H, s, OH), 8.38 (1H, s, OH), 6.58 (1H, d, J = 3 Hz, H6′), 6.52 (1H, d, J = 8.5 Hz, H3′), 6.48 (1H, d, J = 8.5 Hz, H3), 6.36 (1H, d, J = 3 Hz, 8.5 Hz, H4′), 6.34 (1H, d, J = 3 Hz, H6), 6.25 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 5.26 (1H, d, J = 6 Hz, NH), 3.99 (2H, d, J = 6 Hz, CH_2N), 3.52 (3H, s, CH_3), 3.39 (2H, s, CH_2). Anal. ($C_{16}H_{17}NO_5$) C, H, N.

Reduction with $NaBH_4$ in Methanol: Method b_3. 2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methyl]amino]-*N*-(benzyloxy)phenylacetyl Amide (II_5). To a solution of I_5 (200 mg, 0.59 mmol) in 10 mL of methanol was added 20 mg (0.53 mmol) of sodium borohydride. The reaction mixture was maintained at room temperature for 15 min. The solution was neutralized to pH = 7 with 6 N HCl. The residue obtained by evaporating off the solvent on a rotary evaporator was added to 10 mL of water and extracted three times with ethyl acetate. The combined extracts were washed with water and finally dried over anhydrous sodium sulfate. The solvent was then evaporated, and the residue was purified by flash chromatography on silica gel (9:1, dichloromethane/methanol) to provide 189 mg (90%) of II_5: mp 151–153 °C; ^1H NMR (DMSO) δ 11.08 (1H, s, CONH), 8.68 (1H, s, OH), 8.48 (1H, s, OH), 7.3 (5H, s, Ph), 6.6 (1H, d, J = 3 Hz, H6′), 6.52 (1H, d, J = 8.5 Hz, H3′), 6.48 (1H, d, J = 8.5 Hz, H3), 6.36 (1H, d, J = 3 Hz, 8.5 Hz, H4′), 6.35 (1H, d, J = 3 Hz, H6), 6.23 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 5.28 (1H, d, J = 6 Hz, NH), 4.75 (2H, s, CH_2), 3.98 (4H, d, J = 6 Hz, CH_2N), 3.1 (2H, s, CH_2CO_2). Anal. ($C_{22}H_{22}N_2O_5$) C, H, N.

Reduction with Fe/HCl in Aqueous Ethanol: Method b_4. 5-Amino-2-hydroxybenzoic Acid 3-Phenylprop-2-en-1-yl Ester (B_{30}). A solution of A_{30} (2.99 g, 10 mmol), 1.68 g (30 mmol) of powdered iron, and 5 mL of 37% aqueous HCl was refluxed in 200 mL of 10% aqueous sodium hydrogen carbonate. Insoluble material was filtered off. Ethanol was removed in vacuo, and the residue was extracted twice with ethyl acetate, washed with brine, dried with sodium sulfate, and evaporated to dryness. Purification, by flash chromatography on silica gel (7:3, cyclohexane: ethyl acetate), gave 650 mg (24%): mp 141 °C; ^{1}H NMR (DMSO) δ 9.82 (bs, 1H, OH), 7.55 (2H, d, J = 8 Hz, H2′, H6′), 7.40 (2H, t, J = 8 Hz, H3′, H5′), 7.33 (1H, t, J = 8 Hz, H4′), 7.12 (1H, d, J = 8.5 Hz, H6), 6.88 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 6.85 (1H, d, J = 16 Hz, CH-Ph), 6.76 (1H, d, J = 8.5 Hz, H3), 6.53 (1H, dt, J = 6 Hz, 16 Hz, CH_2-CH=), 5.01 (2H, d, J = 8 Hz, CO_2-CH_2), 4.85 (bs, 2H, NH_2).

Reduction with Aqueous $Na_2S_2O_4$: Method b_5. 2-Hydroxy-5-amino-*N*-(benzyloxy)phenylacetyl Amide (B_5). To a solution of A_5 (500 mg, 1.66 mmol, 1 equiv) in 22 mL of 10% aqueous ammonia was added by portion 2 g of sodium hydrosulfite (11.49 mmol, 6.9 equiv). The reaction gave a white precipitate on standing at room temperature for 30 min, which was collected, washed with water, and dried to give 328 mg (73%) of B_5: mp 150–151 °C; ^{1}H NMR (DMSO) δ 11.05 (1H, s, NH), 8.5 (1H, s, OH), 7.3 (5H, s, Ph), 6.46 (1H, d, J = 8.5 Hz, H3), 6.32 (1H, d, J = 3 Hz, H6), 6.26 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 4.75 (2H, s, CH_2Ph), 4.35 (2H, s, NH_2), 3.1 (2H, s, CH_2).

General Procedures of Esterification: Method c_1. 2-Hydroxy-5-nitrophenylacetic Acid Methyl Ester (A_2). To a solution of A_1 (500 mg, 2.54 mmol, 2 equiv) in 10 mL of methanol was added dropwise at 0 °C 1 mL of thionyl chloride (13.7 mmol, 5.4 equiv). The solution was heated under reflux for 6 h. The solvent was stripped off on a rotary evaporator. The residue was then dissolved in 25 mL of ethyl acetate. The solvent was washed sequentially with 10% $NaHCO_3$, water, 1 N aqueous hydrochloride, and saturated aqueous sodium chloride and dried over anhydrous sodium sulfate. The solid obtained by stripping off the solvent on a rotary evaporator was purified by recrystallization from ethanol and water to provide 390 mg (69%) of A_1: mp 155.5–156.5 °C; ^{1}H NMR (DMSO) δ 11.2 (1H, s, OH), 8.1 (1H, d, J = 3 Hz, H6), 8.0 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 6.9 (1H, d, J = 8.5 Hz, H3), 3.68 (2H, s, CH_2), 3.55 (3H, s, CH_3).

Method c_2. 2-Hydroxy-5-nitrobenzoic Acid *tert*-Butyl Ester (A_{15}). A mixture of 5-nitrosalicylic acid (1 g, 5.46 mmol, 1 equiv), *tert*-butyl alcohol (0.405 g, 5.46 mmol, 1 equiv), dicyclohexylcarbodiimide (DCC) (1.24 g, 6.01 mmol, 1.1 equiv), and dimethylaminopyridine (DMAP) (0.1 g, 0.55 mmol, 0.1 equiv) in 20 mL of ethyl ether and 10 mL of tetrahydrofuran (THF) was stirred for 2 days, until the reaction was judged complete by TLC analysis, and then filtered. The solvent was removed in vacuo. The residue was dissolved in ethyl acetate, washed sequentially with 1 N ammonia and saturated aqueous sodium chloride, dried over anhydrous sodium sulfate, and concentrated. Purification by flash chromatography over silica gel (1:1, ethyl acetate:hexane) provided 0.81 g (62%) of the desired compound A_{15}: mp 81–81.5 °C; ^{1}H NMR (DMSO) δ 11.5 (1H, s, OH), 8.4 (1H, d, J = 3 Hz, H6), 8.27 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 7.08 (1H, d, J = 8.5 Hz, H3), 1.55 (9H, s, $C(CH_3)_3$).

Method c_3. 2-Hydroxy-5-nitrobenzoic Acid 2-(1-Tricyclo[3.3.1.1]decyl)ethyl Ester (A_{22}). A suspension of 2-hydroxy-5-nitrobenzoic acid (9.16 g, 50 mmol) and thionyl chloride (36.5 mL, 0.5 mmol) in 100 mL of dry toluene was heated at 80 °C, until gas evolution stopped and complete dissolution occurred. Excess of thionyl chloride and toluene were removed in vacuo, and the crude acid chloride was dissolved in 500 mL of dry THF. To this solution was slowly added a solution of lithium 2-(1-tricyclo[3.3.1.1]decyl)ethanolate, freshly prepared from 9 g (50 mmol) of alcohol in 250 mL of dry THF and 31.5 mL (50 mmol) of *n*-butyllithium (1.6 M in hexane). After 18 h of continuous stirring at room temperature, THF was concentrated in vacuo and the residue was dissolved in dichloromethane, washed sequentially with 1 N HCl and water, dried with sodium sulfate, and evaporated to dryness. Purification by flash chromatography over silica gel (7.5:2.5, cyclohexane:ethyl acetate) gave 10.5 g (61%) of A_{22}: mp 106 °C; ^{1}H NMR (DMSO) δ 8.55 (1H, d, J = 3 Hz, H6), 8.32 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 7.25 (1H, d, J = 8.5 Hz, H3), 4.4 (2H, J = 7 Hz, CO_2CH_2), 2.0–1.5 (18H, m, CH_2 and CH from tricyclo[3.3.1.1]decyl).

Method c_4. 2-Hydroxy-5-nitrobenzoic Acid 3-Hydroxyphenyl Ester (A_{33}). To a solution of poly(2-hydroxy-5-nitrobenzoate) (18.3 g, 0.1 mmol), prepared in 70% yield from 2-hydroxy-5-nitrobenzoic acid and DCC in pyridine and *N*,*N*-dimethylformamide (DMF) according to Steward[51] and resorcinol (11 g, 0.1 mmol) in 500 mL of dichloromethane, was added imidazole (68 g, 1 mmol) by portions at room temperature. The resulting solution was stirred at room temperature until completion of the reaction (TLC). After sequential washing with water, 1 N HCl, and water, the organic layer was dried with sodium sulfate and evaporated to dryness. Purification, by flash chromatography on silica gel (9.5:0.5, dichloromethane:ethyl acetate), gave 12.93 g (47%) of A33: mp 156 °C; ^{1}H NMR (DMSO) δ 9.80 (1H, s, OH), 8.70 (1H, d, J = 3 Hz, H6), 8.38 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 7.27 (1 H, t, J = 8.5 Hz, H5′), 7.21 (1H, d, J = 8.5 Hz, H3), 6.80–6.65 (3H, m, H2′, H4′, H6′).

General Procedure of Reductive Amination: Method d_1. 2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methyl]amino]benzoic Acid *n*-hexadecyl Ester (II_{20}). A solution of 5-amino-2-hydroxybenzoic acid *n*-hexadecyl ester (B_{20}) (2.6 g, 10 mmol) and 2,5-dihydroxybenzaldehyde (1.47 g, 10 mmol) was refluxed in 50 mL of methanol, until completion of the reaction (2 h, monitored by TLC), and then cooled. To this cooled solution was added sodium cyanoborohydride (1.26 g, 20 mmol), and stirring was continued until completion of the reaction (18 h, monitored by TLC). The solution was then hydrolyzed with 1 N HCl and brine and the crude compound extracted twice with ethyl acetate, washed with water, dried with sodium sulfate, and evaporated to dryness. Purification, by flash chromatography on silica gel (7:3, cyclohexane:ethyl acetate), gave 2.6 g (45%) of II_{20}: mp 102 °C; ^{1}H NMR (DMSO) δ 11.57 (1H, s, OH), 6.95 (1H, d, J = 3 Hz, H6), 6.85 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 6.70 (1H, d, J = 8.5 Hz, H3), 6.60 (1H, d, J = 3 Hz, H6′), 6.58 (1H, d, J = 8.5 Hz, H3′), 6.33 (1H, dd, J = 3 Hz, 8.5 Hz, H4′), 5.90 (1H, t, J = 6.5 Hz, NH), 4.42 (2H, t, J = 7 Hz, CO_2-CH_2), 4.15 (2H, t, J = 6.5 Hz, CH_2–N), 1.82 (2H, m, CO_2–CH_2-CH_2), 1.45–1.20 (26H, m, $(CH_2)_{13}$-CH_3), 0.87 (3H, t, J = 7 Hz, CH_3). Anal. ($C_{30}H_{45}NO_5$) C, H, N, O.

Method d_2. 2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methyl]amino]benzoic Acid 4-Phenylphenyl Ester (II_{34}). A solution of 5-amino-2-hydroxybenzoic acid 4-phenylphenyl ester (B_{34}) (1.53 g, 5 mmol) and 2,5-dihydroxybenzaldehyde (0.69 g, 5 mmol) was refluxed in 25 mL of toluene, until completion of the reaction (6 h, monitored by TLC). The cooled solution was evaporated and the residue dissolved in 150 mL of dichloromethane and hydrogenated with 250 mg of 5% Pd/C at room temperature and atmospheric pressure, until consumption of the theoretical amount of hydrogen. Catalyst was filtered off and solvent concentrated in vacuo. Purification, by flash chromatography on silica gel (7:3, cyclohexane:ethyl acetate), gave 1.70 g (79%) of II_{34}: mp 190 °C; ^{1}H NMR (DMSO) δ 9.55 (1H, s, OH), 8.82 (1H, s, OH), 8.62 (1H, s, OH), 7.77–7.43 (9H, m, 4-phenylphenyl), 7.20 (1H, d, J = 3.5 Hz, H6), 6.98 (1H, dd, J = 3.5 Hz, 9 Hz, H4), 6.84 (1H, d, J = 9 Hz, H3), 6.70 (1H, d, J = 3 Hz, H6′), 6.63 (1H, d, J = 8.5 Hz, H3′), 6.48 (1H, dd, J = 3 Hz, 8.5 Hz, H4′), 5.92 (1H, t, J = 6.5 Hz, NH), 4.13 (2H, d, J = 6.5 Hz, CH_2). Anal. ($C_{26}H_{21}NO_5$) C, H, N, O.

General Procedure of Phosphonate Hydrolysis: Method e. [[2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methyl]amino]-phenyl]methyl]phosphonic Acid Hydrochloride (II_7). I_7 (170 mg) was dissolved in 5 mL of 6 N HCl. The solution was heated to 120 °C and then maintained overnight. The solvent was then evaporated and the residue dissolved in water, treated with activated charcoal, and lyophilized to give 118 mg (73%) of II_7: mp > 300 °C; ^{1}H NMR (DMSO) δ 10.6 (2H, bs, OH), 10.0 (2H, bs, NH_2), 9.4 (1H, bs, OH), 8.8 (1H, s, OH), 7.3 (1H, d, J = 3 Hz, H6), 7.08 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 6.82 (1H, d, J = 8.5 Hz, H3), 6.7 (1H, d, J = 8.5 Hz, H3′), 6.66 (1H, d, J = 3 Hz, H6′), 6.58 (1H, dd, J = 3 Hz, 8.5 Hz, H4′), 4.15 (2H, s, CH_2N), 2.9 (2H, d, J = 22 Hz, CH_2P). Anal. ($C_{14}H_{17}NO_6PCl$) C, H, N.

General Procedure of Nitration: Method f. 2-Hydroxy-5-nitrophenylacetic Acid (A_1). To a solution of 2-hydroxyphenylacetic acid (6.08 g) in 16 mL of water was added dropwise,

under stirring at 0 °C, 8 mL of nitric acid (40%). After the addition, the reaction mixture was maintained for 1.5 h at 5 °C and allowed to warm to 25 °C, and stirring was maintained for 30 min more. It was then poured into water with ice. The precipitate was collected, washed with water, and recrystallized with ethanol and water to give 2-hydroxy-3-nitrophenylacetic acid (2.85 g, 36%) and 3.37 g (43%) of A_1: mp 159.5–160.5 °C; ^{1}H NMR (DMSO) δ 12.3 (1H, s, CO_2H), 11.1 (1H, s, OH), 8.1 (1H, d, J = 3 Hz, H6), 8.0 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 6.9 (1H, d, J = 8.5 Hz, H3), 3.55 (2H, s, CH_2).

General Procedure of Hydroxylamine Condensation: Method g. 2-Hydroxy-5-nitro-*N*-*tert*-butoxyphenylacetyl Amide (A_4). To a solution of *N*-*tert*-butoxyamine hydrochloride (1.21 g, 9.63 mmol, 1 equiv) in 16 mL of anhydrous chloroform were added at 0 °C 1.4 mL of triethylamine (10 mmol, 1.04 equiv), a solution of A_1 (1.9 g, 9.64 mmol, 1 equiv) in 40 mL of anhydrous THF, and dicyclohexylcarbodiimide (1.1 equiv). The reaction mixture was stirred at room temperature for 2 days and then filtered. The solvent was evaporated on a rotary evaporator. The residue was dissolved in ethyl acetate, washed sequentially with 1 N aqueous hydrochloride, water, 1 N ammonia, and saturated aqueous sodium chloride, dried over anhydrous sodium sulfate, and concentrated. Purification by flash chromatography over silica gel (dichloromethane) gave 2.15 g (83%) of A_4: mp 182–184 °C; ^{1}H NMR (DMSO) δ 10.48 (1H, s, NH), 8.05 (1H, d, J = 3 Hz, H6), 8.0 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 6.9 (1H, d, J = 8.5 Hz, H3), 3.4 (2H, s, CH_2), 1.1 (9H, s $C(CH_3)_3$).

General Procedure of Phosphonate Preparation: Method h. [(2-Hydroxy-5-nitrophenyl)methyl]phosphonic Acid Diethyl Ester (A_6). A mixture of 2-hydroxy-5-nitrobenzyl chloride (1 g) in 4 mL of triethyl phosphite was stirred at 60 °C for 4 h. After cooling, the white precipitate was collected and dried to give 1.54 g (100%) of A_6: mp 139–140 °C; ^{1}H NMR (DMSO) δ 8.08 (1H, d, J = 3 Hz, H6), 7.95 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 6.9 (1H, d, J = 8.5 Hz, H3), 3.9 (4H, q, J = 8.5 Hz, CH_2), 3.2 (2H, d, J = 21 Hz, CH_2P), 1.12 (6H, t, J = 8.5 Hz, CH_3).

General Procedure of Amino-Group Protection: Method i. 2-Hydroxy-5-[(*tert*-butoxycarbonyl)amino]benzoic Acid (C_{12}). To a mixture of 5-aminosalicylic acid (13.77 g, 90 mmol, 1 equiv) in 240 mL of dioxane and 120 mL of water was added triethylamine (18 g, 180 mmol, 2 equiv) followed by di-*tert*-butyl dicarbonate (21.6 g, 180 mmol, 2 equiv). The reaction was stirred at room temperature for 3 h. Solvent were removed in vacuo, and 3 N aqueous hydrochloride was added dropwise to the residue. A precipitate was obtained, collected, washed with water, and dried to provide 22 g (97%) of C_{12}: mp 278 °C; ^{1}H NMR (DMSO) δ 9.2 (1H, s, NH), 7.9 (1H, d, J = 3 Hz, H6), 7.45 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 6.8 (1H, d, 8.5 Hz, H3), 1.3 (9H, s, $C(CH_3)_3$).

General Procedure of Amino-Group Deprotection: Method j. 2-Hydroxy-5-amino-*N*-(benzyloxy)benzoyl Amide Trifluoroacetic Acid (B_{11}). A solution of B_{11} (1.5 g, 4.19 mmol) in 12 mL of 2:1 dichloromethane:trifluoroacetic acid was stirred at room temperature for 2 h. The solvent was evaporated in vacuo. The residue was then added in ether. The precipitate was collected, washed with ether, and dried to provide 1.4 g (90%) of B_{11}: mp 200–201 °C; ^{1}H NMR (DMSO) δ 11.4 (1H, s, CONH), 10.0 (3H, s, NH_3), 7.5 (1H, d, J = 3 Hz, H6), 7.35 (5H, m, Ph), 7.2 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 6.95 (1H, d, 8.5 Hz, H3), 4.9 (2H, s, CH_2).

2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methylidene]amino]phenylacetic Acid Methyl ester (I_2). B_2 (269 mg, 1.49 mmol, 1 equiv) and 2,5-dihydroxybenzaldehyde (206 mg, 1.49 mmol, 1 equiv) were coupled according to method a. Purification by flash chromatography over silica gel (20:1, dichloromethane:methanol) gave 359 mg (80%) of I_2: mp 139.5–140.5 °C; ^{1}H NMR (DMSO) δ 12.25 (1H, s, OH), 9.7 (1H, s, OH), 9.1 (1H, s, OH), 8.75 (1H, s, CH=N), 7.2 (1H, d, J = 3 Hz, H6), 7.18 (1 H, dd, J = 3 Hz, 8.5 Hz, H4), 6.95 (1 H, d, J = 3 Hz, H6′), 6.8 (1H, d, J = 8.5 Hz, H3), 6.78 (1 H, dd, J = 3 Hz, 8.5 Hz, H4′), 6.70 (1H, d, J = 8.5 Hz, H3′), 3.58 (5H, s, CH_2, CH_3). Anal. ($C_{16}H_{15}NO_5$) C, H, N.

2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methylidene]amino]phenylacetic Acid Ethyl Ester (I_3). B_3 (380 mg, 1.95 mmol, 1 equiv) and 2,5-dihydroxybenzaldehyde (269 mg, 1.95 mmol, 1 equiv) were coupled according to method a. Purification by flash chromatography over silica gel (20:1, dichloromethane: methanol) gave 419 mg (68%) of I_3: mp 183–184 °C; ^{1}H NMR (DMSO) δ 12.25 (1H, s, OH), 9.8 (1H, s, OH), 9.1 (1H, s, OH), 8.72 (1H, s, CH=N), 7.2 (1H, d, J = 3 Hz, H6), 7.18 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 6.9 (1H, d, J = 3 Hz, H6′), 6.8 (1H, d, J = 8.5 Hz, H3), 6.78 (1H, dd, J = 3 Hz, 8.5 Hz, H4′), 6.70 (1H, d, J = 8.5 Hz, H3′), 4.05 (2H, q, J = 8.5 Hz, CO_2CH_2), 3.55 (2H, s, CH_2P), 1.15 (3H, t, J = 8.5 Hz, CH_3). Anal. ($C_{17}H_{17}NO_5$) C, H. N.

2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methylidene]amino]-*N*-*tert*-butoxyphenylacetyl Amide (I_4). B_4 (238 mg, 1 mmol, 1 equiv) and 2,5-dihydroxybenzaldehyde (138 mg, 1 mmol, 1 equiv) were coupled according to method a. Purification by flash chromatography over silica gel (9:1, dichloromethane;methanol) gave 188 mg (53%) of I_4: mp 190.5–191.5 °C; ^{1}H NMR (DMSO) δ 12.25 (1H, s, OH), 10.4 (1H, s, NH), 9.8 (1H, s, OH), 9.0 (1H, s, OH), 8.8 (1H, s, CH=N), 7.17 (1H, d, J = 3 Hz, H6), 7.15 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 6.9 (1H, d, J = 3 Hz, H6′), 6.8 (1H, d, J = 8.5 Hz, H3), 6.75 (1H, dd, J = 3 Hz, 8.5 Hz, H4′), 6.70 (1H, d, J = 8.5 Hz, H3′), 3.3 (2H, s, CH_2), 1.25 (9H, s, $C(CH_3)_3$). Anal. ($C_{19}H_{22}N_2O_5$) C, H, N.

2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methylidene]amino]-*N*-[(phenylmethyl)oxy]phenylacetyl Amide (I_5). B_5 (300 mg, 1.10 mmol, 1 equiv) and 2,5-dihydroxybenzaldehyde (152 mg, 1.10 mmol, 1 equiv) were coupled according to method a. Purification by flash chromatography over silica gel (9:1, dichloromethane:methanol) gave 323 mg (75%) of I_5: mp 210–211 °C; ^{1}H NMR (DMSO) δ 12.55 (1H, s, OH), 11.0 (1H, s, NH), 9.8 (1H, s, OH), 9.0 (1H, s, OH), 8.7 (1H, s, CH=N), 7.3 (5H, m, Ph), 7.17 (1H, d, J = 3 Hz, H6), 7.15 (1H, dd, J = 3 Hz, 8. 5 Hz, H4), 6.9 (1H, d, J = 3 Hz, H6′), 6.8 (1H, d, J = 8.5 Hz, H3), 6.75 (1H, dd, J = 3 Hz, 8.5 Hz, H4′), 6.70 (1H, d, J = 8.5 Hz, H3′), 4.8 (2H, s, CH_2O), 3.3 (2H, s, CH_2CO). Anal. ($C_{22}H_{20}N_2O_5$) C, H, N.

[[2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methylidene]amino]phenyl]methyl]phosphonic Acid Diethyl Ester (I_6). B_6 (337 mg, 1.30 mmol, 1 equiv) and 2,5-dihydroxybenzaldehyde (180 mg, 1.30 mmol, 1 equiv) were coupled according to method a. Purification by flash chromatography over silica gel (dichloromethane) gave 271 mg (55%) of I_6: mp 138–139 °C; ^{1}H NMR (DMSO) δ 12.45 (1H, s, OH), 9.75 (1H, s, OH), 9.0 (1H, s, OH), 8.7 (1H, s, CH=N), 7.2 (1H, d, J = 3 Hz, H6), 7.16 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 6.9 (1H, d, J = 3 Hz, H6′), 6.82 (1H, d, J = 8.5 Hz, H3), 6.78 (1H, dd, J = 3 Hz, 8.5 Hz, H4′), 6.70 (1H, d, J = 8.5 Hz, H3′), 3.9 (4H, d, J – 8.5 Hz, OCH_2), 3.12 (2H, d, J = 20 Hz, CH_2P), 1.15 (6H, t, J = 8.5 Hz, CH_3). Anal. ($C_{18}H_{22}NO_6P$) C, H, N.

2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methylidene]amino]-*N*-methoxybenzoyl Amide (I_9). B_9 (390 mg, 1.32 mmol, 1 equiv) and 2,5-hydroxybenzaldehyde (182 mg, 1.32 mmol, 1 equiv) were coupled according to method a. Purification by flash chromatography over silica gel (9:1, dichloromethane:methanol) gave 250 mg (63%) of I_9: mp 222–224 °C; ^{1}H NMR (DMSO) δ 12.2 (1H, s, OH), 11.85 (1 H, s, NH), 9.05 (1H, s, OH), 8.8 (1H, s, CH=N), 7.7 (1H, d, J = 3 Hz, H6), 7.5 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 6.97 (2H, d, J = 8.5 Hz, H3), 6.95 (1H, d, J = 3 Hz, H6′), 6.8 (1H, dd, J = 3 Hz, 8.5 Hz, H4′), 6.72 (1H, d, J = 8.5 Hz, H3′), 3.7 (3H, s, CH_3). Anal. ($C_{15}H_{14}N_2O_5$) C, H, N.

2-Hydroxy-5-[(2,5-dihydroxyphenyl)methylidene]amino]-*N*-*tert*-butoxybenzoyl Amide (I_{10}). B_{10} (250 mg, 1.12 mmol, 1 equiv) and 2,5-dihydroxybenzaldehyde (154 mg, 1.12 mmol, 1 equiv) were coupled according to method a. Purification by flash chromatography over silica gel (9:1, dichloromethane:methanol) gave 319 mg (83%) of I_{10}: mp 252–253 °C; ^{1}H NMR (DMSO) δ 12.25 (1H, s, OH), 11.8 (1H, s, NH), 11.1 (1H, s, OH), 9.05 (1H, s, OH), 8.8 (1H, s, CH=N), 7.73 (1H, d, J = 3 Hz, H6), 7.5 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 6.97 (2H, d, J = 8.5 Hz, H3), 6.95 (1H, d, J = 3 Hz, H6′), 6.78 (1H, dd, J = 3 Hz, 8.5 Hz, H4′), 6.72 (1H, d, J = 8.5 Hz, H3′), 1.25 (9H, s, $C(CH_3)_3$). Anal. ($C_{16}H_{20}N_2O_5$) C, H, N.

2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methylidene]amino]-*N*-(benzyloxy)benzoyl Amide (I_{11}). B_{11} (295 mg, 1 mmol, 1 equiv) and 2,5-dihydroxybenzaldehyde (138 mg, 1 mmol, 1 equiv) were coupled according to method a. The precipitate was collected, washed with methanol, and dried to give 314 mg (83%) of I_{11}: mp 220–221 °C; ^{1}H NMR (DMSO) δ 12.24 (1H, s, OH), 11.8 (1H, s, NH), 9.05 (1H, s, OH), 8.8 (1H, s, CH=N), 7.68 (1H,

d, J = 3 Hz, H6), 7.5 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 7.48–7.3 (5H, m, Ph), 6.97 (2H, d, J = 8.5 Hz, H3), 6.95 (1H, d, J = 3 Hz, H6′), 6.8 (1H, dd, J = 3 Hz, 8.5 Hz, H4′), 6.72 (1H, d, J = 8.5 Hz, H3′), 4.9 (2H, s, CH_2). Anal. ($C_{21}H_{18}N_2O_5$) C, H, N.

2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methylidene]amino]benzoic Acid (I_{12}). 5-Aminosalicylic acid (1.11 g, 7.24 mmol, 1 equiv) and 2,5-dihydroxybenzaldehyde (1 g, 7.24 mmol, 1 equiv) were coupled in DMF according to method a. Purification by flash chromatography over silica gel (4:1, dichloromethane:methanol) gave 1.55 g (78%) of I_{12}; mp >300 °C dec; ^{1}H NMR (DMSO) δ 12.65 (1H, s, OH), 11.8 (1H, s, OH), 9.02 (1H, s, OH), 8.8 (1H, s, CH=N), 7.75 (1H, d, J = 3 Hz, H6), 7.3 (1 H, dd, J = 3 Hz, 8.5 Hz, H4), 6.95 (1H, d, J = 3 Hz, H6′), 6.7 (3H, m, H3, H4′, H3′). Anal. ($C_{14}H_{11}NO_5$) C, H, N.

2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methylidene]amino]benzoic Acid Methyl Ester (I_{13}). B_{13} (476 mg, 2.29 mmol, 1 equiv) and 2,5-dihydroxybenzaldehyde (317 mg, 2.30 mmol, 1 equiv) were coupled according to method a. The precipitate was collected, washed, with methanol, and dried to give 539 mg (82%) of I_{13}: mp 221.5-222.5 °C; ^{1}H NMR (DMSO) δ 12.15 (1 H, s, OH), 10.49 (1H, s, OH), 9.05 (1H, s, OH), 8.8 (1H, s, CH=N), 7.75 (1H, d, J = 3 Hz, H6), 7.6 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 7.02 (1H, d, J = 8.5 Hz, H3), 6.99 (1H, d, J = 3 Hz, H6′), 6.8 (1H, dd, J = 3 Hz, 8.5 Hz, H4′), 6.72 (1H, d, J = 8.5 Hz, H3′), 3.9 (3H, s, CH_3). Anal. ($C_{16}H_{13}NO_5$) C, H, N.

2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methylidene]amino]benzoic Acid Ethyl Ester (I_{14}). B_{14} (500 mg, 2.3 mmol) and 2,5-dihydroxybenzaldehyde (317 mg, 2.3 mmol) were coupled according to method a. The precipitate was collected, washed with ethanol, and dried to give 447 mg (65%) of I_{14}: mp 185–186 °C; ^{1}H NMR (DMSO) δ 12.1 (1H, s, OH), 10.52 (1H, s, OH), 9.05 (1H, s, OH), 8.8 (1H, s, CH=N), 7.7 (1H, d, J = 3 Hz, H6), 7.6 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 7.02 (1H, d, J = 8.5 Hz, H3), 6.99 (1H, d, J = 3 Hz, H6′), 6.8 (1H, dd, J = 3 Hz, 8.5 Hz, H4′), 6.72 (1H, d, J = 8.5 Hz, H3′), 4.35 (2H, q, J = 8.5 Hz, CH_2), 1.32 (3H, t, J = 8.5 Hz, CH_3). Anal. ($C_{16}H_{15}NO_5$) C, H, N.

2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methylidene]amino]benzoic Acid *tert*-Butyl Ester (I_{15}). B_{15} (300 mg, 1.44 mmol, 1 equiv) and 2,5-dihydroxybenzaldehyde (200 mg, 1.45 mmol, 1 equiv) were coupled according to method a. Purification by flash chromatography over silica gel (dichloromethane) gave 450 mg (95%) of I_{15}. An analytical sample was prepared by recrystallization from ethyl acetate and hexane: mp 168.5–169.5 °C; ^{1}H NMR (DMSO) δ 12.1 (1H, s, OH), 10.65 (1H, bs, OH), 9.02 (1H, bs, OH), 8.8 (1H, s, CH=N), 7.65 (1H, d, J = 3 Hz, H6), 7.6 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 7.01 (1H, d, J = 8.5 Hz, H3), 6.9 (1H, s, J = 3 Hz, H6′), 6.8 (1H, dd, J = 3 Hz, 8.5 Hz, H4′), 6.73 (1H, d, J = 8.5 Hz, H3′), 1.58 (9H, s, $C(CH_3)_3$). Anal. ($C_{18}H_{19}NO_5$) C, H, N.

2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methylidene]amino]benzoic Acid 3,3-Dimethylbutyl Ester (I_{16}). B_{16} (500 mg, 1.86 mmol, 1 equiv) and 2,5-dihydroxybenzaldehyde (258 mg, 1.87 mmol, 1 equiv) were coupled according to method a. Crystallization of the residue with methanol afforded 470 mg (65%) of I_{16}: mp 160.5–161.5 °C; ^{1}H NMR (DMSO) δ 12.1 (1H, s, OH), 10.5 (1H, bs, OH), 9.1 (1H, bs, OH), 8.78 (1H, s, CH=N), 7.7 (1H, d, J = 3 Hz, H6), 7.6 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 7.02 (1H, d, J = 8.5 Hz, H3), 6.9 (1H, d, J = 3 Hz, H6′), 6.8 (1H, dd, J = 3 Hz, 8.5 Hz, H4′), 6.72 (1H, d, J = 8.5 Hz, H3′), 4.35 (2H, t, J = 8 Hz, CO_2CH_2), 1.65 (2H, t, J = 8 Hz, CH_2), 0.95 (9H, s, $C(CH_3)_3$). Anal. ($C_{20}H_{23}NO_5$) C, H, N.

2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methylidene]amino]benzoic Acid 2,4,4-Trimethylpentyl Ester (I_{17}). B_{17} (460 mg, 1.74 mmol, 1 equiv) and 2,5-dihydroxybenzaldehyde (240 mg, 1.74 mmol, 1 equiv) were coupled according to method a. Purification by flash chromatography over silica gel (dichloromethane) gave 505 mg (76%) of I_{17}; mp 91–92.5 °C; ^{1}H NMR (DMSO) δ 12.1 (1H, s, OH), 9.1 (1H, s, OH), 8.78 (1H, s, CH=N), 7.69 (1H, d, J = 3 Hz, H6), 7.6 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 7.0 (1H, d, J = 8.5 Hz, H3), 6.98 (1H, d, J = 3 Hz, H6′), 6.79 (1H, dd, J = 3 Hz, 8.5 Hz, H4′), 6.72 (1H, d, J = 8.5 Hz, H3′), 4.15–3.95 (2H, m, CO_2CH_2), 1.95 (1H, m, CH), 1.48–1.0 (2H, m, CH_2), 0.98 (3H, d, J = 8.5 Hz, CH_3), 0.88 (9H, s, $C(CH_3)_3$. Anal. ($C_{22}H_{27}NO_5$) C, H, N.

2-Hydroxy-5[*N*-[(2,5-dihydroxyphenyl)methylidene]amino]benzoic Acid 3,5,5-Trimethylphenyl Ester (I_{18}). B_{18} (1.5 g, 5.38 mmol, 1 equiv) and 2,5-dihydroxybenzaldehyde (743 mg, 5.38 mmol, 1 equiv) were coupled according to method a. Purification by flash chromatography over silica gel (dichloromethane) gave 2.12 g (99%) of I_{18}: mp 130–131 °C; ^{1}H NMR (DMSO) δ 12.1 (1H, s, OH), 9.1 (1H, s, OH), 8.78 (1H, s, CH=N), 7.68 (1H, d, J = 3 Hz, H6), 7.6 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 7.0 (1H, d, J = 8.5 Hz, H3), 6.95 (1H, d, J = 3 Hz, H6′), 6.79 (1H, dd, J = 3 Hz, 8.5 Hz, H4′), 6.7 (1H, d, J = 8.5 Hz, H3′), 4.3 (2H, t, J = 8 Hz, CO_2CH_2), 1.65 (3H, m, CH, CH_2), 1.3–1.0 (2H, m, CH_2), 0.94 (3H, d, J = 8 Hz, CH_3), 0.84 (9H, s, $C(CH_3)_3$). Anal. ($C_{23}H_{29}NO_5$), C, H, N.

2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methylidene]amino]benzoic Acid 3,7-Dimethyloctyl Ester (I_{19}). B_{19} (400 mg, 1.37 mmol, 1 equiv) and 2,5-dihydroxybenzaldehyde (189 mg, 1.37 mmol, 1 equiv) were coupled according to method a. Purification by flash chromatography over silica gel (dichloromethane) gave 553 mg (98%) of I_{19}: mp 120–121 °C; ^{1}H NMR (DMSO) δ 12.08 (1H, s, OH), 10.5 (1H, bs, OH), 9.1 (1H, s, OH), 8.78 (1H, s, CH=N), 7.68 (1H, d, J = 3 Hz, H6), 7.6 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 7.0 (1H, d, J = 8.5 Hz, H3), 6.95 (1H, d, J = 3 Hz, H6′), 6.78 (1H, dd, J = 3 Hz, 8.5 Hz, H4′), 6.72 (1H, d, J = 8.5 Hz, H3′), 4.35 (2H, t, J = 8 Hz, CO_2CH_2), 1.75 (1H, m, CH), 1.5 (3H, m, CH, CH_2), 1.35–1.0 (6H, m, CH_2), 0.9 (3H, d, J = 8 Hz, CH_3), 6 H, d, J = 8 Hz, CH_3). Anal. ($C_{24}H_{31}NO_5$) C, H, N.

2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methylidene]amino]benzoic Acid Cyclohexylmethyl Ester (I_{21}). B_{21} (375 mg, 1.51 mmol, 1 equiv) and 2,5-dihydroxybenzaldehyde (210 mg, 1.51 mmol, 1 equiv) were coupled according to method a. Purification by flash chromatography over silica gel (9:1, dichloromethane:methanol) and recrystallization from dichloromethane gave 550 mg (100%) of I_{21}: mp 123–125 °C; ^{1}H NMR (DMSO) δ 12.09 (1H, s, OH), 10.55 (1H, s, OH), 9.04 (1H, s, OH), 8.8 (1H, s, CH=N), 7.7 (1H, d, J = 3 Hz, H6), 7.6 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 7.05 (1H, d, J = 8.5 Hz, H3), 7.0 (1H, d, J = 3 Hz, H6′), 6.8 (1H, dd, J = 3 Hz, 8.5 Hz, H4′), 6.72 (1H, d, J = 8 Hz, H3′), 4.14 (2H, d, J = 8.5 Hz, CO_2CH_2), 1.8–0.9 (11H, m, C_6H_{11}). Anal. ($C_{21}H_{23}NO_5$) C, H, N.

2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methylidene]amino]benzoic Acid 2-Phenylethyl Ester (I_{25}). B_{25} (210 mg, 0.79 mmol, 1 equiv) and 2,5-dihydroxybenzaldehyde (110 mg, 0.79 mmol, 1 equiv) were coupled according to method a. Purification by recrystallization from chloroform and hexane gave 250 mg (82%) of I_{25}: mp 185–186 °C; ^{1}H NMR (DMSO) δ 12.1 (1H, s, OH), 9.1 (1H, bs, OH), 8.78 (1H, s, CH=N), 7.65 (1H, d, J = 3 Hz, H6), 7.6 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 7.05 (1H, d, J = 8.5 Hz, H3), 6.98 (1H, s, H6′), 6.8 (1H, dd, J = 3 Hz, 8 Hz, H4′), 6.72 (1H, d, J = 8 Hz, H3′), 5.92 (2H, s, CH_2), 1.15 (9H, s, $C(CH_3)_3$. Anal. ($C_{22}H_{19}NO_5$) C, H, N.

2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methylidene]amino]benzoic Acid 3-Phenylpropyl Ester (I_{26}). B_{26} (1 g, 3.69 mmol, 1 equiv) and 2,5-dihydroxybenzaldehyde (510 mg, 3.69 mmol, 1 equiv) were coupled according to method a. The reaction mixture was concentrated. The precipitate was filtered to provide 1.25 g (87%) of I_{26}: mp 170 °C; ^{1}H NMR (DMSO) δ 12.15 (1H, s, OH), 9.1 (1H, s, OH), 8.78 (1H, s, CH=N), 7.28–7.08 (5H, m, Ph), 7.61 (1H, d, J = 3 Hz, H6), 7.6 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 7.0 (1H, d, J = 8.5 Hz, H3), 6.98 (1H, J = 3 Hz, s, H6′), 6.8 (1H, dd, J = 3 Hz, 8.5 Hz, H4′), 6.74 (1H, d, J = 8.5 Hz, H3′), 4.26 (2H, t, J = 8 Hz, CO_2CH_2), 2.7 (2H, t, J = 8 Hz, CH_2Ph), 2.02 (2H, p, J = 8 Hz, CH_2). Anal. ($C_{23}H_{21}NO_5$) C, H, N.

2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methylidene]amino]benzoic Acid 4-Phenylbutyl Ester (I_{27}). B_{27} (1 g, 3.5 mmol, 1 equiv) and 2,5-dihydroxybenzaldehyde (480 mg, 3.5 mmol, 1 equiv) were coupled according to method a. The reaction mixture was concentrated. The precipitate was collected and dried to provide 1.21 g (85%) of I_{27}: mp 127–128 °C; ^{1}H NMR (DMSO) δ 12.15 (1H, s, OH), 10.5 (1H, s, OH), 9.1 (1H, s, OH), 8.78 (1H, s, CH=N), 7.68 (1H, d, J = 3 Hz, H6), 7.6 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 7.28–7.05 (5H, m, Ph), 7.0 (1H, d, J = 8.5 Hz, H3), 6.98 (1H, s, H6′), 6.8 (1H, dd, J = 3 Hz, 8.5 Hz, H4′), 6.72 (1H, d, J = 8.5 Hz, H3′), 4.3 (2H, t, OCH_2), 2.6 (2H, t, CH_2), 1.7 (4H, m, CH_2CH_2Ph). Anal. ($C_{24}H_{23}NO_5$) C, H, N.

2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methylidene]amino]benzoic Acid 2-Methyl-3-phenylpropyl Ester (I_{28}). B_{28} (500 mg, 1.75 mmol, 1 equiv) and 2,5-dihydroxybenzaldehyde

(240 mg, 1.75 mmol, 1 equiv) were coupled according to method a. Purification by recrystallization from dichloromethane gave 500 mg (71%) of I_{28}: mp 132–133 °C; ^{1}H NMR (DMSO) δ 12.1 (1H, s, OH), 10.5 (1H, s, OH), 9.1 (1H, s, OH), 8.78 (1H, s, CH=N), 7.6 (2H, m, H6, H4), 7.28=7.05 (5H, m, Ph), 7.0 (1H, d, J = 8.5 Hz, H3), 6.98 (1H, J = 3 Hz, s, H6′), 6.78 (1H, dd, J = 3 Hz, 8.5 Hz, H4′), 6.72 (1H, d, J = 8.5 Hz, H3′), 4.15 (2H, m, OCH_2), 2.8–2.5 (2H, m, CH_2), 2.2 (1H, m, CH), 0.95 (3H, d, J = 8 Hz, CH_3). Anal. ($C_{24}H_{23}NO_5$) C, N, N.

2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methylidene]amino]benzoic Acid 3-Methyl-3-phenylpropyl Ester (I_{29}). B_{29} (1 g, 3.5 mmol, 1 equiv) and 2,5-dihydroxybenzaldehyde (0.48 g, 3.5 mmol, 1 equiv) were coupled according to method a. Purification by recrystallization from chloroform and hexane gave 1.4 g (99%) of I_{29}: mp 108–110 °C; ^{1}H NMR (DMSO) δ 12.12 (1H, s, OH), 10.5 (1H, s, OH), 9.08 (1H, s, OH), 8.78 (1H, s, CH=N), 7.58 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 7.5 (1H, d, J = 3 Hz, H6), 7.28–7.02 (5H, m, Ph), 6.99 (1H, d, J = 8.5 Hz, H3), 6.98 (1H, d, J = 3 Hz, H6′), 6.8 (1H, dd, J = 3 Hz, 8.5 Hz, H4′), 6.74 (1H, d, J = 8.5 Hz, H3′), 4.15 (2H, m, CO_2CH_2), 2.88 (1H, m, CH), 2.0 (2H, m, CH_2), 1.2 (3H, d J = 8 Hz, CH_3). Anal. ($C_{24}H_{23}NO_5$) C, H, N.

2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methylidene]amino]benzoic Acid *p*-Isopropylbenzyl Ester (I_{31}). B_{31} (400 mg, 1.4 mmol, 1 equiv) and 2,5-dihydroxybenzaldehyde (190 mg, 1.4 mmol, 1 equiv) were coupled according to method a. Purification by flash chromatography over silica gel (100:1, dichloromethane:methanol) and recrystallization from petroleum ether gave 570 mg (100%) of I_{31}: mp 181–182 °C; ^{1}H NMR (DMSO) δ 12.1 (1H, s, OH), 9.03 (1H, bs, OH), 8.8 (1H, s, CH=N), 7.7 (1H, d, J = 3 Hz, H6), 7.6 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 7.38 (2H, d, J = 8.5 Hz, H2″, H6″), 7.22 (2H, d, J = 8.5 Hz, H3″, H5″), 7.02 (1H, d, J = 8.5 Hz, H3), 6.9 (1H, d, J = 3 Hz, H6′), 6.8 (1H, dd, J = 3 Hz, 8.5 Hz, H4′), 6.73 (1H, d, J = 8.5 Hz, H3′), 5.32 (2H, s, CH_2), 2.87 (1H, m, CH), 1.18 (6H, d, J = 8 Hz, CH_3). Anal. ($C_{24}H_{23}NO_5$) C, H, N.

2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methylidene]amino]benzoic Acid 3,5-Dimethylbenzyl Ester (I_{32}). B_{32} (250 mg, 1.84 mmol, 1 equiv) and 2,5-dihydroxybenzaldehyde (250 mg, 1.84 mmol, 1 equiv) were coupled according to method a. Purification by flash chromatography over silica gel (9:1, dichloromethane:methanol) and recrystallization from methanol afforded 650 mg (90%) of I_{32}: mp 187–188 °C; ^{1}H NMR (DMSO) δ 12.1 (1H, s, OH), 9.1 (1H, s, OH), 8.8 (1H, s, CH=N), 7.7 (1H, d, J = 3 Hz, H6), 7.6 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 7.1–6.9 (5H, m, H2″, H6″, H3, H6′, H4″), 6.8 (1H, dd, J = 3 Hz, 8.5 Hz, H4′), 6.72 (1H, d, J = 8.5 Hz, H3′), 5.28 (2H, s, CH_2), 2.22 (6H, s, $(CH_3)_2$). Anal. ($C_{23}H_{21}NO_5$) C, H, N.

2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methyl]amino]phenylacetic Acid Hydrochloride (II_1): prepared from I_1 (200 mg, 0.73 mmol) according to method b_1. The solvent was then evaporated. The residue was dissolved in ethyl acetate and a little of methanol and then filtered. The solution was added with HCl (g) in ethyl acetate. The precipitate formed was collected, washed with ethyl acetate, and dried to give 183 mg of the crude product (80%) of II_1. An analytical sample was prepared by recrystallization from ethyl acetate and methanol: mp >240 °C dec; ^{1}H NMR (DMSO) δ 8.9 (1H, s, OH), 8.59 (1H, s, OH), 6.7–6.5 (5H, m, H6, H4, H3, H6′, H3′), 6.42 (1H, dd, J = 3 Hz, 8.5 Hz, H4′), 4.08 (2H, s, CH_2N), 3.35 (2H, s, CH_2). Anal. ($C_{18}H_{19}NO_5Cl$) C, H, N.

2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methyl]amino]phenylacetic Acid Ethyl Ester (II_3): prepared from I_3 (100 mg, 0.32 mmol) according to method b_2. Purification by flash chromatography on silica gel (9:1, dichloromethane:methanol) provided 90 mg (89%) of II_3: mp 124.5–125.5 °C; ^{1}H NMR (DMSO) δ 8.64 (1H, s, OH), 8.45 (1H, s, OH), 8.38 (1H, s, OH), 6.58 (1H, d, J = 3 Hz, H6′), 6.52 (1H, d, J = 8.5 Hz, H3′), 6.48 (1H, d, J = 8.5 Hz, H3), 6.36 (1H, dd, J = 3 Hz, 8.5 Hz, H4′), 6.34 (1H, d, J = 3 Hz, H6), 6.25 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 5.26 (1H, d, J = 6 Hz, NH), 3.98 (4H, m, CH_2N, CH_2), 3.38 (2H, s, CH_2CO_2), 1.15 (3H, t, J = 8.5 Hz, CH_3). Anal. ($C_{17}H_{19}NO_5$) C, H, N.

2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methyl]amino]-*N*-*tert*-butoxyphenylacetyl Amide Hydrochloride (II_4): prepared from I_4 (200 mg, 0.56 mmol) according to method b_1. Purification by flash chromatography on silica gel (9:1 , dichloromethane:methanol) provided 155 mg (77%) of II_4: mp 109–111 °C; ^{1}H NMR (DMSO) δ 11.1 (1H, s, CONH), 8.7 (1H, s, OH), 8.48 (1H, s, OH), 6.6 (1H, d, J = 3 Hz, H6′), 6.5 (1H, d, J = 8.5 Hz, H3′), 6.5 (1H, d, J = 8.5 Hz, H3), 6.36 (1H, dd, J = 3 Hz, 8.5 Hz, H4′), 6.37 (1H, d, J = 3 Hz, H6), 6.25 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 4.0 (2H, s, CH_2N), 3.3 (2H, s, CH_2CO_2), 1.12 (9H, s, $C(CH_3)_3$. Anal. ($C_{19}H_{24}N_2O_5$) C, H, N.

[2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methyl]amino]benzyl]phosphonic Acid Diethyl Ester (II_6): prepared from I_6 (200 mg, 0.53 mmol) according to method b_1. Purification by flash chromatography on silica gel (9:1, dichloromethane:methanol) provided 173 mg (86%) of II_6: mp 124–125 °C; ^{1}H NMR (DMSO) δ 8.68 (1H, s, OH), 8.46 (1H, s, OH), 8.42 (1H, s, OH), 6.59 (1H, d, J = 3 Hz, H6′), 6.54 (1H, d, J = 8.5 Hz, H3′), 6.5 (1H, d, J = 8.5 Hz, H3), 6.4 (1H, d, J = 3 Hz, H6), 6.37 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 6.27 (1H, dd, J = 3 Hz, 8.5 Hz, H4′), 5.3 (2H, t, J = 3 Hz, NH), 3.98 (2H, d, J = 3 Hz, CH_2N), 3.85 (4H, q, J = 8.5 Hz, CH_2), 2.98 (2H, d, J = 22 Hz, CH_2P), 1.1 (6H, t, J = 8.5 Hz, CH_3). Anal. ($C_{17}H_{24}NO_6P$) C, H, N.

[2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methyl]amino]phenyl]hydroxamic Acid (II_8): prepared from I_8 (650 mg, 1.72 mg) according to method b_1. Purification by recrystallization from ethyl acetate and hexane gave 369 mg (74%) of II_8: mp 180.5–181.5 °C; ^{1}H NMR (DMSO) δ 11.15 (1H, s, OH), 9.15 (1H, s, CONH), 8.7 (1H, s, OH), 8.5 (1H, s, OH), 6.85 (1H, d, J = 3 Hz, H6), 6.69 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 6.67 (1H, d, J = 8.5 Hz, H3), 6.59 (1H, d, J = 3 Hz, H6′), 6.55 (1H, d, J = 8.5 Hz, H3′), 6.38 (1H, dd, J = 3 Hz, 8.5 Hz, H4′), 5.4 (1H, d, J = 6 Hz, NH), 4.0 (2H, d, J = 6 Hz, CH_2). Anal. ($C_{14}H_{14}N_2O_5$) C, H, N.

2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methyl]amino]-*N*-methoxybenzoyl Amide (II_9): prepared from I_9 (100 mg, 0.33 mmol) according to method b_1. Purification by flash chromatography on silica gel (9:1, dichloromethane:methanol) provided 81 mg (80%) of II_9: mp 120–122 °C; ^{1}H NMR (DMSO) δ 11.15 (1H, s, CONH), 8.7 (1H, s, OH), 8.5 (1H, s, OH), 6.82 (1H, d, J = 3 Hz, H6), 6.69 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 6.67 (1H, d, J = 8.5 Hz, H3), 6.59 (1H, d, J = 3 Hz, H6′), 6.55 (1H, d, J = 8.5 Hz, H3′), 6.38 (1H, dd, J = 3 Hz, 8.5 Hz, H4′), 5.5 (1H, d, J = 6 Hz, NH), 4.02 (2H, d, J = 6 Hz, CH_2N), 3.65 (3H, s, CH_3). Anal. ($C_{15}H_{16}N_2O_5$) C, H, N.

2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methyl]amino]-*N*-*tert*-butoxybenzoyl Amide (II_{10}): prepared from I_{10} (200 mg, 0.58 mmol) according to method b_1. Purification by flash chromatography on silica gel (9:1, dichloromethane:methanol) provided 141 mg (70%) of II_{10}: mp 114–116 °C; ^{1}H NMR (DMSO) δ 11.1 (1H, s, CONH), 8.7 (1H, s, OH), 8.5 (1H, s, OH), 6.8 (1H, d, J = 3 Hz, H6), 6.7 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 6.67 (1H, d, J = 8.5 Hz, H3), 6.6 (1H, d, J = 3 Hz, H6′), 6.55 (1H, d, J = 8.5 Hz, H3′), 6.38 (1H, dd, J = 3 Hz, 8.5 Hz, H4′), 5.48 (1H, d, J = 6 Hz, NH), 4.02 (2H, d, J = 6 Hz, CH_2N), 1.02 (9H, s, $C(CH_3)_3$. Anal. ($C_{18}H_{22}H_2O_5$) C, H, N.

2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methyl]amino]-*N*-(benzyloxy)benzoyl Amide (II_{11}): prepared from I_{11} (650 mg, 1.72 mmol) according to method b_3. Purification by flash chromatography on silica gel (9:1, dichloromethane:methanol) provided 634 mg (97%) of II_{11}: mp 154–156 °C; ^{1}H NMR (DMSO) δ 11.15 (1H, s, CONH), 8.7 (1H, s, OH), 8.5 (1H, s, OH), 7.35 (5H, m, Ph), 6.85 (1H, d, J = 3 Hz, H6), 6.69 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 6.67 (1H, d, J = 8.5 Hz, H3), 6.59 (1H, d, J = 3 Hz, H6′), 6.55 (1H, d, J = 8.5 Hz, H3′), 6.38 (1H, dd, J = 3 Hz, 8.5 Hz, H4′), 5.5 (1H, d, J = 6 Hz, NH), 4.9 (2H, s, CH_2), 4.02 (2H, d, J = 6 Hz, CH_2N). Anal. ($C_{21}H_{20}N_2O_5$) C, H, N.

2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methyl]amino]benzoic Acid Hydrochloride (II_{12}): prepared from I_{12} (300 mg, 1.05 mmol) according to method b_1. After the solvent was evaporated, the residue was dissolved in ethyl acetate with a little of methanol and filtered. To this solution was added HCl (g) in ethyl acetate. The precipitate was collected, washed with ethyl acetate, and dried to give 303 mg of the crude product of II_{12} (89%). An analytical sample was prepared by purification by chromatography on LH-20 (1:1, methanol:water): mp >245 °C dec; ^{1}H NMR (DMSO) δ 10.5 (1H, s, OH), 8.8 (1H, s, OH), 8.5 (1H, s, OH), 6.9 (1H, d, J = 3 Hz, H6), 6.8 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 6.67 (1H, d, J = 8.5 Hz, H3), 6.52 (1H, d, J = 3 Hz,

H6'), 6.5 (1H, d, J = 8.5 Hz, H3'), 6.34 (1H, dd, J = 3 Hz, 8.5 Hz, H4'), 4.0 (2H, s, CH_2). Anal. ($C_{14}H_{13}NO_5$·HCl) C, H, N.

2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methyl]amino]-benzoic Acid Methyl Ester (II_{13}): prepared from I_{13} (500 mg, 1.74 mmol) according to method b_1. The precipitate was collected, washed with methanol, and dried to give 388 mg (77%) of I_{13}: mp 194–195 °C; ^{1}H NMR (DMSO) δ 9.75 (1H, s, OH), 8.7 (1H, s, OH), 8.5 (1H, s, OH), 6.9 (1H, d, J = 3 Hz, H6), 6.85 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 6.7 (1H, d, J = 8.5 Hz, H3), 6.6 (1H, d, J = 3 Hz, H6'), 6.55 (1H, d, J = 8.5 Hz, H3'), 6.38 (1H, dd, J = 3 Hz, 8.5 Hz, H4'), 5.75 (1H, s, NH), 4.05 (2H, s, CH_2), 3.82 (3H, s, CH_3). Anal. ($C_{15}H_{15}NO_5$) C, H, N.

2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methyl]amino]-benzoic Acid Ethyl Ester (II_{14}): prepared from I_{14} (100 mg, 0.32 mmol) according to method c_1. Purification by flash chromatography on silica gel (9:1, dichloromethane:methanol) provided 53 mg (55%) of II_{14}: mp 176–177 °C; ^{1}H NMR (DMSO) δ 9.78 (1H, s, OH), 8.68 (1H, s, OH), 8.5 (1H, s, OH), 6.9 (1H, d, J = 3 Hz, H6), 6.8 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 6.7 (1H, d, J = 8.5 Hz, H3), 6.6 (1H, d, J = 3 Hz, H6'), 6.55 (1H, d, J = 8.5 Hz, H3'), 6.38 (1H, dd, J = 3 Hz, 8.5 Hz, H4'), 5.75 (1H, t d, J = 6 Hz, NH), 4.3 (2H, q, J = 8.5 Hz, CH_2), 4.0 (2H, d, J = 6 Hz, CH_2N), 1.28 (3H, t, J = 8.5 Hz, CH_3). Anal. ($C_{16}H_{17}NO_5$) C, H, N.

2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methyl]amino]-benzoic Acid *tert*-Butyl Ester (II_{15}): prepared from I_{15} (100 mg, 0.3 mmol) according to method b_2. Purification by flash chromatography on silica gel (dichloromethane) provided 55 mg (52%) of II_{15}: mp 115–116 °C; ^{1}H NMR (DMSO) δ 9.9 (1H, s, OH), 8.7 (1H, s, OH), 8.5 (1H, s, OH), 6.89 (1H, d, J = 3 Hz, H6), 6.78 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 6.66 (1H, d, J = 8.5 Hz, H3), 6.58 (1H, d, J = 3 Hz, H6'), 6.55 (1H, d, J = 8.5 Hz, H3'), 6.38 (1H, dd, J = 3 Hz, 8.5 Hz, H4'), 5.7 (1H, d, J = 6 Hz, NH), 4.0 (2H, d, J = 6 Hz, CH_2), 1.5 (9H, s, $C(CH_3)_3$). Anal. ($C_{18}H_{21}NO_5$) C, H, N.

2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methyl]amino]-benzoic Acid 3,3-Dimethylbutyl Ester (II_{16}): prepared from I_{16} (50 mg, 0.13 mmol) according to method b_2. Purification by flash chromatography on silica gel (dichloromethane) provided 46 mg (91%) of II_{16}: mp 127–128 °C; ^{1}H NMR (DMSO) δ 9.8 (1H, s, OH), 8.68 (1H, s, OH), 8.45 (1H, s, OH), 6.88 (1H, d, J = 3 Hz, H6), 6.82 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 6.7 (1H, d, J = 8.5 Hz, H3), 6.57 (1H, d, J = 3 Hz, H6'), 6.55 (1H, d, J = 8.5 Hz, H3'), 6.38 (1H, dd, J = 3 Hz, 8.5 Hz, H4'), 5.75 (1H, d, J = 6 Hz, NH), 4.3 (2H, t, J = 8.5 Hz, CO_2CH_2), 4.0 (2H, d, J = 6 Hz, CH_2N), 1.6 (2H, t, J = 8.5 Hz, CH_2), 0.9 (9H, s, $C(CH_3)_3$). Anal. ($C_{20}H_{25}NO_5$) C, H, N.

2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methyl]amino]-benzoic Acid 2,4,4-Trimethylpentyl Ester (II_{17}): prepared from I_{17} (60 mg, 0.16 mmol) according to method b_2. Purification by flash chromatography on silica gel (9:1, dichloromethane: methanol) provided 50 mg (83%) of II_{17}: mp 119–121 °C; ^{1}H NMR (DMSO) δ 9.8 (1H, s, OH), 8.67 (1H, s, OH), 8.45 (1H, s, OH), 6.89 (1H, d, J = 3 Hz, H6), 6.85 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 6.7 (1H, d, J = 8.5 Hz, H3), 6.55 (1H, d, J = 3 Hz, H6'), 6.53 (1H, d, J = 8.5 Hz, H3'), 6.38 (1H, dd, J = 3 Hz, 8.5 Hz, H4'), 5.75 (1H, d, J = 6 Hz, NH), 4.1–3.88 (2H, m, CO_2CH_2), 4.0 (2H, d, J = 6 Hz, CH_2N), 1.88 (1H, m, CH), 1.3–0.96 (2H, m, CH_2), 0.92 (3H, d, J = 8.5 Hz, CH_3), 0.85 (9H, s, $C(CH_3)_3$). Anal. ($C_{22}H_{29}NO_5$) C, H, N.

2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methyl]amino]-benzoic Acid 3,5,5-Trimethylhexyl Ester (II_{18}): prepared from I_{18} (100 mg, 0.25 mmol) according to method b_2. Purification by flash chromatography on silica gel (9:1, dichloromethane: methanol) provided 76 mg (76%) of II_{18}: mp 103–104 °C; ^{1}H NMR (DMSO) δ 9.8 (1H, s, OH), 8.6 (1H, s, OH), 6.89 (1H, d, J = 3 Hz, H6), 6.82 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 6.7 (1H, d, J = 8.5 Hz, H3), 6.57 (1H, d, J = 3 Hz, H6'), 6.55 (1H, d, J = 8.5 Hz, H3'), 6.38 (1H, dd, J = 3 Hz, 8.5 Hz, H4'), 5.7 (1H, d, J = 6 Hz, NH), 4.25 (2H, m, CO_2CH_2), 4.0 (2H, d, J = 6 Hz, CH_2N), 1.6 (3H, m, CH, CH_2), 1.26–0.98 (2H, m, CH_2), 0.9 (3H, d, J = 8.5 Hz, CH_3), 0.8 (9H, s, $C(CH_3)_3$). Anal. ($C_{23}H_{31}NO_5$) C, H, N.

2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methyl]amino]-benzoic Acid 3,7-Dimethyloctyl Ester (II_{19}): prepared from I_{19} (150 mg, 0.36 mmol) according to method b_2. Purification by flash chromatography on silica gel (9:1, dichloromethane:methanol) provided 130 mg (86%) of II_{19}: mp 97–98 °C; ^{1}H NMR (DMSO) δ 9.78 (1H, s, OH), 8.67 (1H, s, OH), 8.48 (1H, s, OH), 6.89 (1H, d, J = 3 Hz, H6), 6.8 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 6.7 (1H, d, J = 8.5 Hz, H3), 6.57 (1H, d, J = 3 Hz, H6'), 6.54 (1H, d, J = 8.5 Hz, H3'), 6.38 (1H, dd, J = 3 Hz, 8.5 Hz, H4'), 5.72 (1H, d, J = 6 Hz, NH), 4.28 (2H, t, J = 8.5 Hz, CO_2CH_2), 4.0 (2H, d, J = 6 Hz, CH_2N), 1.68 (1H, m, CH), 1.48 (3H, m, CH_2, CH), 1.3–1.0 (6H, m, CH_2), 0.85 (3H, d, J = 8.5 Hz, CH_3), 0.8 (6H, d, J = 8.5 Hz, CH_3). Anal. ($C_{24}H_{33}NO_5$) C, H, N.

2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methyl]amino]-benzoic Acid Cyclohexylmethyl Ester (II_{21}): prepared from I_{21} (500 mg, 1.36 mmol) according to method b_2. Purification by flash chromatography on silica gel (50:1, dichloromethane: methanol) and then recrystallization from methanol provided 322 mg (64%) of II_{21}: mp 167 °C; ^{1}H NMR (DMSO) δ 9.78 (1H, s, OH), 8.65 (1H, s, OH), 8.5 (1H, s, OH), 7.35 (5H, m, Ph), 6.9 (1H, d, J = 3 Hz, H6), 6.81 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 6.7 (1H, d, J = 8.5 Hz, H3), 6.57 (1H, d, J = 3 Hz, H6'), 6.56 (1H, d, J = 8.5 Hz, H3'), 6.38 (1H, dd, J = 3 Hz, 8.5 Hz, H4'), 5.85 (1H, d, J = 6 Hz, NH), 4.04 (4H, d, CH_2, CH_2N), 1.8–0.85 (11H, m, C_6H_{11}). Anal. ($C_{21}H_{25}NO_5$) C, H, N.

5-[*N*-[(2,5-Dihydroxyphenyl)methyl]amino]-2-hydroxybenzoic Acid 2-(1-Tricyclo[3.3.1.1]decyl)ethyl Ester (II_{22}): prepared from B_{22} (3.16 g, 10 mmol) and 2,5-dihydroxybenzaldehyde in methanol (1.47 g, 10 mmol) followed by reduction with sodium cyanoborohydride in methanol according to method d_1. Purification, by flash chromatography on silica gel (7.5:2.5, cyclohexane:ethyl acetate), gave 1.15 g (26%) of II_{22}: mp 151 °C; ^{1}H NMR (DMSO) δ 9.30 (1H, s, OH), 8.75 (1H, s, OH), 8.55 (1H, s, OH), 6.93 (1H, d, J = 3.5 Hz, H6), 6.88 (1H, dd, J = 3.5 Hz, 9 Hz, H4), 6.75 (1H, d, J = 9 Hz, H3), 6.62 (1H, d, J = 3 Hz, H6'), 6.60 (d, J = 8.5 Hz, H3'), 6.33 (1H, dd, J = 3 Hz, 8.5 Hz, H4'), 5.82 (1H, t, J = 6.5 Hz, NH), 4.35 (2H, t, J = 6.5 Hz, CO_2CH_2), 4.05 (2H, d, J = 6.5 Hz, CH_2N), 1.92 (2H, m, CH_2-tricyclo[3.3.1.1]-decyl), 1.70–1.45 (15H, m, tricyclo[3.3.1.1.]decyl). Anal. ($C_{26}H_{31}NO_5$) C, H, N, O.

5-[*N*-[(2,5-Dihydroxyphenyl)methyl]amino]-2-hydroxybenzoic Acid Phenyl Ester (II_{23}): prepared from B_{23} (1.15 g, 5 mmol) and 2,5-dihydroxybenzaldehyde in methanol (0.69 g, 5 mmol) followed by reduction with sodium cyanoborohydride in methanol according to method d_1. Purification, by flash chromatography on silica gel (9.5:0.5, dichloromethane:ethyl acetate), gave 0.32 g (18%) of II_{23}: mp 132 °C; ^{1}H NMR (DMSO) δ 9.52 (1H, s, OH), 8.78 (1H, s, OH), 8.58 (1H, s, OH), 7.48 (2H, t, J = 8.5 Hz, H3'', H5''), 7.32 (1H, t, J = 8.5 Hz, H4''), 7.27 (2H, d, J = 8.5 Hz, H2'', H6''), 7.14 (1H, d, J = 3 Hz, H6), 6.93 (1H, dd, J = 3 Hz, 9 Hz, H4), 6.82 (1H, d, J = 9 Hz, H3), 6.65 (1H, d, J = 3 Hz, H6'), 6.60 (1H, d, J = 8.5 Hz, H3'), 6.43 (1H, dd, J = 3.5 Hz, 8.5 Hz, H4'), 5.90 (1H, t, J = 6 Hz, NH), 4.12 (2H, t, J = 6 Hz, CH_2). Anal. ($C_{20}H_{17}NO_5$) C, H, N, O.

5-[*N*-[(2,5-Dihydroxyphenyl)methyl]amino]-2-hydroxybenzoic Acid Phenylmethyl Ester (II_{24}): prepared from B_{24} (135 mg, 0.55 mmol) and 2,5-dihydroxybenzaldehyde in methanol (76 mg, 0.55 mmol) followed by reduction with sodium cyanoborohydride in methanol according to method d_1. Purification, by flash chromatography on silica gel (9.6:0.4, dichloromethane:ethyl acetate), gave 76 mg (38%) of II_{24}: mp 124 °C; ^{1}H NMR (DMSO) δ 10.30 (1H, m, OH), 7.50–7.25 (5H, m, phenyl), 7.38 (1H, d, J = 8 Hz, H6), 7.02 (1H, dd, J = 3 Hz, 9 Hz, H4), 6.89 (1H, d, J = 9 Hz, H3), 6.75 (1H, d, J = 8.5 Hz, H3'), 6.65 (1H, dd, J = 3 Hz, 8.5 Hz, H4'), 6.61 (1H, d, J = 3 Hz, H6'), 5.37 (2H, s, CO_2CH_2), 4.29 (2H, s, CH_2-NH). Anal. ($C_{21}H_{19}NO_5$) C, H, N, O.

2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methyl]amino]-benzoic Acid 2-Phenylethyl Ester (II_{25}): prepared from I_{25} (150 mg, 0.4 mmol) according to method b_2. Purification by flash chromatography on silica gel (dichloromethane) provided 137 mg (91%) of II_{25}: mp 185–186 °C; ^{1}H NMR (DMSO) δ 9.7 (1H, s, OH), 8.72 (1H, s, OH), 8.48 (1H, s, OH), 7.3–7.1 (5H, m, Ph), 6.84 (1H, d, J = 3 Hz, H6), 6.82 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 6.7 (1H, d, J = 8.5 Hz, H3), 6.58 (1H, d, J = 3 Hz, H6'), 6.55 (1H, d, J = 8.5 Hz, H3'), 6.38 (1H, dd, J = 3 Hz, 8.5 Hz, H4'), 5.7 (1H, t, J = 6 Hz, NH), 4.4 (2H, t, J = 8.5 Hz, CO_2CH_2), 4.02 (2H, d, J = 6 Hz, CH_2N), 2.95 (2H, t, J = 8.5 Hz, CH_2). Anal. ($C_{22}H_{21}NO_5$) C, H, N.

2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methyl]amino]-benzoic Acid 3-Phenylpropyl Ester (II_{26}): prepared from I_{26} (1 g, 2.56 mmol) according to method b_2. Purification by flash chromatography on silica gel (50:1, dichloromethane:methanol) and then recrystallization from ethyl acetate and petroleum ether provided 0.6 g (60%) of II_{26}: mp 142 °C; ^{1}H NMR (DMSO) δ 9.78 (1H, s, OH), 8.72 (1H, s, OH), 8.5 (1H, s, OH), 7.28–7.1 (5H, m, Ph), 6.94 (1H, d, J = 3 Hz, H6), 6.82 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 6.7 (1H, d, J = 8.5 Hz, H3), 6.58 (1H, d, J = 3 Hz, H6′), 6.55 (1H, d, J = 8.5 Hz, H3′), 6.35 (1H, dd, J = 3 Hz, 8.5 Hz, H4′), 5.8 (1H, t, J = 6 Hz, NH), 4.22 (2H, t, J = 8 Hz, CO_2CH_2), 4.04 (2H, d, J = 6 Hz, CH_2N), 2.64 (2H, t, J = 8 Hz, CH_2Ph), 1.96 (2H, p, J = 8 Hz, CH_2). Anal. ($C_{23}H_{23}NO_5$) C, H, N.

4-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methyl]amino]-benzoic Acid 4-Phenylbutyl Ester (II_{27}): prepared from I_{27} (600 mg, 1.48 mmol) according to method b_2. Purification by flash chromatography on silica gel (50:1, dichloromethane: methanol) and then recrystallization from dichloromethane and hexane provided 392 mg (65%) of II_{27}: mp 143 °C; ^{1}H NMR (DMSO) δ 9.78 (1H, s, OH), 8.68 (1H, s, OH), 8.48 (1H, s, OH), 7.28–7.1 (5H, m, Ph), 6.9 (1H, d, J = 3 Hz, H6), 6.8 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 6.7 (1H, d, J = 8.5 Hz, H3), 6.59 (1H, d, J = 3 Hz, H6′), 6.55 (1H, d, J = 8.5 Hz, H3′), 6.38 (1H, dd, J = 3 Hz, 8.5 Hz, H4′), 5.75 (1H, d, J = 6 Hz, NH), 4.25 (2H, t, 8 Hz, OCH_2), 4.0 (2H, d, J = 6 Hz, CH_2N), 2.6 (2H, t, J = 8 Hz, CH_2Ph), 1.65 (4H, m, CH_2Cl_2). Anal. ($C_{24}H_{25}NO_5$), C, H, N.

2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methyl]amino]-benzoic Acid 2-Methyl-3-phenylpropyl Ester (II_{28}): prepared from I_{28} (500 mg, 1.23 mmol) according to method b_2. Purification by flash chromatography on silica gel (50:1, dichloromethane: methanol) and then recrystallization from dichloromethane and hexane provided 280 mg (56%) of II_{28}: mp 121–122 °C; ^{1}H NMR (DMSO) δ 9.72 (1H, s, OH), 8.68 (1H, s, OH), 8.48 (1H, s, OH), 7.28–7.1 (5H, m, Ph), 6.92 (1H, d, J = 3 Hz, H6), 6.81 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 6.7 (1H, d, J = 8.5 Hz, H3), 6.59 (1H, d, J = 3 Hz, H6′), 6.55 (1H, d, J = 8.5 Hz, H3′), 6.38 (1H, dd, J = 3 Hz, 8.5 Hz, H4′), 5.8 (1H, d, J = 6 Hz, NH), 4.05 (4H, m, OCH_2, CH_2N), 2.7–2.4 (2H, m, CH_2), 2.1 (1H, m, CH), 0.85 (3H, d, J = 8.5 Hz, CH_3). Anal. ($C_{24}H_{25}NO_5$) C, H, N.

2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methyl]amino]-benzoic Acid 3-Methyl-3-phenylpropyl Ester (II_{29}): prepared from I_{29} (700 mg, 1.73 mmol) according to method b_2. Purification by flash chromatography on silica gel (50:1, dichloromethane: methanol) and then recrystallization from dichloromethane and hexane provided 450 mg (64%) of II_{29}: mp 107–108 °C; ^{1}H NMR (DMSO) δ 9.72 (1H, s, OH), 8.7 (1H, s, OH), 8.48 (1H, s, OH), 7.28–7.1 (5H, m, Ph), 6.87 (1H, d, J = 3 Hz, H6), 6.81 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 6.68 (1H, d, J = 8.5 Hz, H3), 6.58 (1H, d, J = 3 Hz, H6′), 6.55 (1H, d, J = 8.5 Hz, H3′), 6.37 (1H, dd, J = 3 Hz, 8.5 Hz, H4′), 5.78 (1H, d, J = 6 Hz, NH), 4.2–3.85 (2H, m, CO_2CH_2), 4.02 (2H, d, J = 6 Hz, CH_2N), 2.8 (1H, m, CH), 1.9 (2H, m, CH_2), 1.2 (3H, d, J = 8.5 Hz, CH_3). Anal. ($C_{24}H_{25}NO_5$) C, H, N.

5-[*N*-[(2,5-Dihydroxyphenyl)methyl]amino]-2-hydroxybenzoic Acid 3-Phenylprop-2-en-1-yl Ester (II_{30}): prepared from I_{30} (1.35 g, 5 mmol) and 2,5-dihydroxybenzaldehyde in toluene (0.69 g, 5 mmol) followed by reduction with sodium cyanoborohydride in toluene according to method d_1. Purification, by flash chromatography on silica gel (9:1, dichloromethane:ethyl acetate), gave 0.69 g (35%) of II_{30}: mp 135 °C; ^{1}H NMR (DMSO) δ 9.85 (1H, s, OH), 8.80 (1H, s, OH), 8.60 (1H, s, OH), 7.55–7.35 (5H, mt, Ph), 7.08 (1H, d, J = 3 Hz, H6), 6.88 (1H, dd, J = 3.5 Hz, 9 Hz, H4), 6.82 (1H, d, J = 16 Hz, =CH-Ph), 6.80 (1H, d, J = 9 Hz, H3), 6.67 (1H, d, J = 3.5 Hz, H6′), 6.65 (1H, d, J = 8.5 Hz, H3′), 6.54 (1H, dt, J = 16 Hz, 6.5 Hz, CH_2-*CH*=), 6.47 (1H, dd, J = 3.5 Hz, 8.5 Hz, H4′), 5.90 (1H, t, J = 6.5 Hz, HN), 5.02 (2H, d, J = 6.5 Hz, CO_2CH_2), 4.12 (2H, d, J = 6.5 Hz, CH_2NH). Anal. ($C_{23}H_{21}NO_5$) C, H, N, O.

2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methyl]amino]-benzoic Acid *p*-Isopropylbenzyl Ester (II_{31}): prepared from I_{31} (500 mg, 1.23 mmol) according to method b_2. Purification by flash chromatography on silica gel (50:1, dichloromethane: methanol) and then recrystallization from dichloromethane and petroleum ether provided 392 mg (78%) of II_{31}: mp 155–156 °C; ^{1}H NMR (DMSO) δ 9.7 (1H, s, OH), 8.72 (1H, s, OH), 8.5 (1H, s, OH), 7.3–7.2 (4H, q, J = 8.5 Hz, H2″, H3″, H5″, H6″), 6.92 (1H, d, J = 3 Hz, H6), 6.8 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 6.7 (1H, d, J = 8.5 Hz, H3), 6.56 (1H, d, J = 3 Hz, H6′), 6.55 (1H, d, J = 8.5 Hz, H3′), 6.38 (1H, dd, J = 3 Hz, 8.5 Hz, H4′), 5.8 (1H, t, J = 6 Hz, NH), 5.25 (2H, s, CH_2), 4.02 (2H, d, J = 6 Hz, CH_2N), 1.18 (3H, d, J = 8.5 Hz, CH_3). Anal. ($C_{24}H_{25}NO_5$) C, H, N.

2-Hydroxy-5-[*N*-[(2,5-dihydroxyphenyl)methyl]amino]-benzoic Acid 3,5-Dimethylbenzyl Ester (II_{32}): prepared from I_{32} (500 mg, 1.28 mmol) according to method b_2. Purification by flash chromatography on silica gel (50:1, dichlomethane:methanol) and then recrystallization from diethyl ether and petroleum ether provided 221 g (44%) of II_{32}: mp 142–143 °C; ^{1}H NMR (DMSO) δ 9.67 (1H, s, OH), 8.7 (1H, s, OH), 8.48 (1H, s, OH), 6.95 (6H, m, Ph, H6), 6.8 (1H, dd, J = 3 Hz, 8.5 Hz, H4), 6.7 (1H, d, J = 8.5 Hz, H3), 6.56 (1H, d, J = 3 Hz, H6′), 6.55 (1H, d, J = 8.5 Hz, H3′), 6.38 (1H, dd, J =3Hz, 8.5 Hz, H4′), 5.8 (1H, t, J = 6 Hz, NH), 5.22 (2H, s, CH_2), 4.03 (2H, d, J = 6 Hz, CH_2N), 2.28 (6H, s, CH_3). Anal. ($C_{23}H_{23}NO_5$) C, H, N.

5-[*N*-[(2,5-Dihydroxyphenyl)methyl]-2-hydroxybenzoic Acid 3-Hydroxyphenyl Ester (II_{33}): prepared from B_{33} (0.74 g, 3 mmol) and 2,5-dihydroxybenzaldehyde in a mixture of toluene and methanol (0.41 g, 3 mmol) followed by catalytic hydrogenation in ethyl acetate according to method d_2. Purification, by flash chromatography on silica gel (5.5:4.5, cyclohexane:ethyl acetate), gave 0.16 g (16%) of II_{33}: mp 113 °C; ^{1}H NMR (DMSO) δ 7.28 (1H, t, J = 8.5 Hz, H5″), 7.17 (1H, d, J = 3.5 Hz, H6), 6.96 (1H, dd, J = 3.5 Hz, 9 Hz, H4), 6.86 (1H, d, J = 9 Hz, H3), 6.80–6.65 (4H, m, H6′, H2″, H4″, H6″), 6.65 (1H, d, J = 8.5 Hz, H3′), 6.48 (1H, dd, J = 3 Hz, 8.5 Hz, H4′), 5.94 (1H, t, J = 6.5 Hz, NH), 4.12 (2H, d, J = 6.5 Hz, CH_2). Anal. ($C_{20}H_{17}NO_6$) C, H, N, O.

5-[*N*-[(2,5-Dihydroxyphenyl)methyl]amino]-2-hydroxybenzoic Acid 1-Naphthyl Ester (II_{35}): prepared from B_{35} (1.12 g, 4 mmol) and 2,5-dihydroxybenzaldehyde in toluene (0.55 g, 4 mmol) followed by catalytic hydrogenation with palladium in dichloromethane according to method d_2. Purification, by flash chromatography on silica gel (9.8:0.2, dichloromethane:ethyl acetate), gave 0.73 g (45%) of II_{35}: mp 145 °C; ^{1}H NMR (DMSO) δ 9.54 (1H, s, OH), 8.76 (1H, s, OH), 8.60 (1H, s, OH), 8.10–7.45 (7H, m, naphthyl), 7.34 (1H, d, J = 3.5 Hz, H6), 7.03 (1H, dd, J = 3.5 Hz, 9 Hz, H4), 6.90 (1H, d, J = 9 Hz, H3), 6.72 (1H, d, J = 3 Hz, H6′), 6.64 (1H, d, J = 8.5 Hz, H3′), 6.47 (1H, dd, J = 3 Hz, 8.5 Hz, H4′), 6.00 (1H, t, J = 6.5 Hz, NH), 4.20 (2H, d, J = 6.5 Hz, CH_2Cl_2). Anal. ($C_{24}H_{19}NO_5$) C, H, N, O.

5-[*N*-[(2,5-Dihydroxyphenyl)methyl]amino]-2-hydroxybenzoic Acid 2-Naphthyl Ester (II_{36}): prepared from B_{36} (1.40 g, 5 mmol) and 2,5-dihydroxybenzaldehyde in toluene (0.69 g, 5 mmol) followed by catalytic hydrogenation with palladium in ethyl acetate according to method d_2. Purification, by flash chromatography on silica gel (7:3, cyclohexane:ethyl acetate), gave 0.94 g (47%) of II_{36}: mp 179 °C; ^{1}H NMR (DMSO) δ 9.55 (1H, s, OH), 8.82 (1H, s, OH), 8.62 (1H, s, OH), 8.05–7.47 (7H, m, naphthyl), 7.22 (1H, d, J = 3.5 Hz, H6), 6.96 (1H, dd, J = 3.5 Hz, 9 Hz, H4), 6.85 (1H, d, J = 9 Hz, H3), 6.67 (1H, d, J = 3 Hz, H6′), 6.62 (1H, d, J = 8.5 Hz, H2, H3′), 6.45 (1H, dd, J = 3 Hz, 8.5 Hz, H4′), 6.02 (1H, t, J = 6.5 Hz, NH), 4.17 (2H, d, J = 6.5 Hz, CH_2). Anal. ($C_{24}H_{19}NO_5$) C, H, N, O.

Cell Cultures. Cells termed ER 22 were prepared by transfecting CCL 39 hamster fibroblasts with wild-type human EGF-receptor to obtain a cell clone exhibiting about 8×10^5 EGF-binding sites/cell. The preparation of the DNA constructs and the characterization of cell lines expressing them were described by G. L.'Allemain.[43]

Cells were routinely grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) containing the antibiotic G418 (200 μg/mL) at 37 °C in 5% CO_2.

DNA Synthesis. Cells were seeded at 3.5×10^5 cells by well in 24-well Nuncion dishes. The cells were grown to confluence in DMEM supplemented with 10% FCS. To obtain quiescent cells, the medium was changed to DMEM/HAM'S F12 (1:1) for 48 h.

The cells were incubated with different concentrations of the inhibitory compounds (dissolved at 1000 × final concentration in DMSO 100%) for 1 h. Then, EGF (20 ng/mL) (Collaborative Biochemical Products, cat 40010) or FCS and 0.1 μCi of methyl-^3H thymidine ([^{3}H]Me-dT, NEN, NET 027Z) were added. The incorporation of thymidine into the trichloroacetic acid insoluble fraction was determined by a scintillation counter.

Membrane Preparation. ER 22 cells were grown in 850-cm^3 tissue culture roller bottles to obtain 10^9 cells, and cell membranes were purified on sucrose 32% (w/w), according to the published procedure of G. Carpenter.[52] Membrane preparations were suspended in Hepes (20 mM, pH 7.4) and $MgCl_2$ (10 mM), aliquoted, and stored frozen at −80 °C.

In Vitro Tyrosine Kinase Assay. The tyrosine kinase assay was performed as previously described.[16] The reaction was carried out in a final volume of 50 μL containing 20 mM Hepes, pH 7.4, 1 mM $MnCl_2$, 0.1 mg/mL BSA, 100 ng/mL EGF, 0.5 mg/mL tridecapeptide (RRLIEDAEYAARG=RR-Src, H5445 Bachem), membrane fraction of ER 22 cells, 5 μM ATP, and 1 μCi of [γ^{32}P]-ATP (NEN, NEG 002H, 3000Ci/mM) with or without inhibitors at various concentrations.

EGF-receptor was first incubated with EGF for 10 min at room temperature; then, the inhibitor was added, and the reaction was initiated by the addition of the peptide and ATP. Incubation was carried out for 20 min at room temperature. The reaction was terminated by addition of 25 μL of trichloroacetic acid 10% in the presence of 10 μL of BSA (10 mg/mL). Precipitated proteins were removed by centrifugation, and 40 μL of the supernatant was spotted on Whatman P81 phosphocellulose papers (2 cm × 2 cm) that were immediately immersed in orthophosphoric acid (75 mM) for 15 min. This operation was repeated three times, and then the papers were dried, and the radioactivity was counted with a scintillation counter.

In Vitro Other Kinases Assays. Protein kinase A was purchased from Sigma (P8164), and the assay was performed as described by M. K. Smith[53] with histone H1-7 (H-1805 Bachem) as substrate. Protein kinase C was prepared on DEAE-cellulose as described by U. Kikkawa (54) from rat brain, and the assay was performed with the Kit PKC Amersham (RPN 77). EGF-receptor was purified on WGA sepharose (L-6257, Sigma) and the autophosphorylation assay performed as described by A. Gazit.[22]

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