The excess of HF was removed in vacuo. The residue was triturated with anhydrous ether and then was dissolved in (1:1:0.01) water–acetonitrile–acetic acid and lyophilized to give the crude peptide. The product was purified with HPLC using a Dynamax C-18 300A 12-μm (21.4 × 250 mm) column equilibrated with 10% acetonitrile/90% water containing 0.1% TFA at a flow rate of 20 mL/min. The peptide was eluted with a linear gradient from 10% to 51% acetonitrile over a period of 50 min. UV detection was run at 260 nM. The product was collected as a single peak. Selected fractions were lyophilized to give over 95% pure product, on the basis of analytical HPLC, FAB mass spectrometry, and amino acid analysis.

Biological Assays. The receptor binding assay was modified from a literature procedure.[31] A rat pituitary plasma membrane fraction was prepared as a source of LH-RH receptors, and ^{125}I-[Tyr^5]leuprolide[34] was made via chloramine-T oxidation and purified by using ion-exchange chromatography. Several concentrations of test compounds were coincubated for 120 min at 4 °C with ^{125}I-[Tyr^5]leuprolide[34] and receptors to achieve equilibrium binding; the bound tracer was separated from free by centrifugation. The ability of compounds to release luteinizing hormone (LH) from cultured pituitary cells[32,33] was utilized to establish their biological activities. Pituitaries were dispersed with collagenase and hyaluronidase, and the cells were plated for 72 h. The cells were incubated with test compounds for 3 h at 37 °C, and then the medium was harvested for determination of released LH via RIA. The RIA utilized ^{125}I-LH (ovine) as a tracer, immunoaffinity-purified anti-rat LH rabbit IgG, and rat LH (NIH-RP2) standard. Anti-LH rabbit IgG was obtained from Dr. P. M. Conn of the University of Iowa. Bound tracer and free tracer were separated with immobilized protein A.

In vivo testing of compounds was conducted in urethane anesthetized (800 mg/kg, ip) mature castrate (30-day) male rats which have 10–20-fold elevated basal plasma LH levels.[39] Compounds were administered by iv infusion for 120 min through the tail vein in 20% propylene glycol in normal saline, at a rate of 1 mL/h. Blood samples of 0.4-mL volume were drawn by jugular venipuncture periodically over the 2-h infusion period and for 4 h after the infusion was discontinued. Plasma samples were assayed for LH by using the same RIA for rat LH described above, except that goat anti-rabbit antibody was used to precipitate bound tracer.

Acknowledgment. We are indebted to Dr. P. Michael Conn of the University of Iowa for providing the anti-rat LH antibody and for his many helpful suggestions regarding biological methodology.

Registry No. 1, 121962-61-6; **2**, 121962-62-7; **3**, 121962-63-8; 4, 121962-64-9; **5**, 121962-65-0; **6**, 121962-66-1; **7**, 121962-67-2; **8**, 121962-68-3; **9**, 121962-69-4; **10**, 121962-70-7; 11, 121962-71-8; **12**, 121962-72-9; **13**, 121962-73-0; 14, 121962-74-1; **15**, 121962-75-2; **16**, 121962-76-3; **17**, 121962-77-4; **18**, 121962-28-5; **19**, 121962-79-6; **20**, 121987-55-1; **21**, 121962-80-9; **22**, 121962-81-0; **23**, 121987-56-2; **24**, 121962-82-1; **25**, 121962-83-2; **26**, 122045-80-1; **27**, 121962-84-3; **28**, 121962-85-4; **29**, 121962-86-5; **30**, 121962-87-6; **31**, 121962-88-7; **32**, 121962-89-8; **33**, 121962-90-1; **34**, 121962-91-2; **35**, 121987-57-3; LH, 9002-67-9.

(39) Romano, M. I.; Machiavelli, G. A.; Alonso, G. E.; Burdman, J. A. *Horm. Metab. Res.* **1986**, *18*, 31.

Tyrphostins I: Synthesis and Biological Activity of Protein Tyrosine Kinase Inhibitors

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A novel class of low molecular weight protein tyrosine kinase inhibitors is described. These compounds constitute a systematic series of molecules with a progressive increase in affinity toward the substrate site of the EGF receptor kinase domain. These competitive inhibitors also effectively block the EGF-dependent autophosphorylation of the receptor. The potent EGF receptor kinase blockers examined were found to competitively inhibit the homologous insulin receptor kinase at 10^2–10^3 higher inhibitor concentrations in spite of the significant homology between these protein tyrosine kinases. These results demonstrate the ability to synthesize selective tyrosine kinase inhibitors. The most potent EGF receptor kinase inhibitors also inhibit the EGF-dependent proliferation of A431/clone 15 cells with little or no effect on EGF independent cell growth. These results demonstrate the potential use of *protein* tyrosine kinase inhibitors as selective antiproliferative agents for proliferative diseases caused by the hyperactivity of protein tyrosine kinases. We have suggested the name "tyrphostins" for this class of antiproliferative compounds which act as protein tyrosine kinase blockers.

Among the numerous oncogene products, many exhibit protein tyrosine kinase (PTK) activity, which is essential for their biological activity (see ref 1 and 2, for review). Similarly, the initial event in mitogenesis induction by plasma growth factors is the ligand-induced autophosphorylation of the cell surface receptor and the phosphorylation of a host of intracellular substrates.[2-9] Similarly, the hormone insulin induces autophosphorylation of its receptor on specific tyrosine residues as well as hormone-induced tyrosine phosphorylation of intracellular target proteins. (See ref 10, for review.) Site-directed mutagenesis within the insulin receptor[10] and the epidermal growth factor (EGF) receptor[11,12] molecules which eliminate their PTK activities nullify their biological activity. These findings, which demonstrate that PTK

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(1) Carpenter, G. *Annu. Rev. Biochem.* **1987**, *56*, 881.
(2) Yarden, Y.; Ulrich, A. *Annu. Rev. Biochem.* **1988** *57*, 443.
(3) Erneaux, C.; Cohen, S.; Garbers, G. L. *J. Biol. Chem.* **1983**, *258*, 4137.
(4) Sawyer, S. T.; Cohen, S. *J. Biol. Chem.* **1985**, *260*, 8233.
(5) Gilmore, T.; Martin, G. S. *Nature* **1983**, *306*, 487.
(6) Bertics, P. J.; Gill, G. N. *J. Biol. Chem.* **1985**, *260*, 14642.
(7) Downward, J.; Waterfield, M. D.; Parker, P. *J. Biol. Chem.* **1985**, *260*, 14538.
(8) Gullick, W. J.; Downward J.; Waterfield, M. D. *EMBO J.* **1985**, *4*, 2869.
(9) Cohen, S.; Fara, R. A. *J. Biol. Chem.* **1985**, *260*, 12351.
(10) Rosen, O. M. *Science* **1987**, *237*, 1452.
(11) Honegger, A. M.; Szopary, D.; Schmidt, A.; Lyall, R.; Van Oberghen, E.; Dull, T. J.; Ulrich, A.; Schlessinger, M. *J. Mol. Cell. Biol.* **1987**, *7*, 4568.

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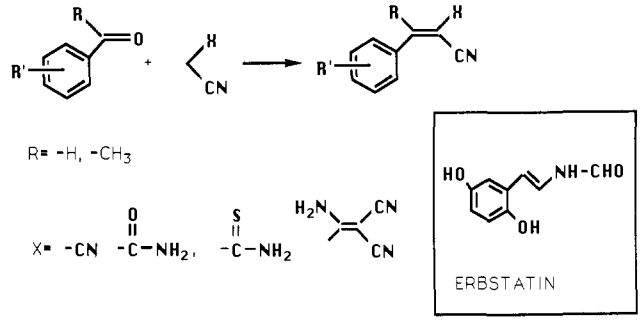
activity of these molecules is essential for their signalling action, led us to the hypothesis that low molecular weight inhibitors, termed "tyrphostins", designed to bind to the substrate subsite of the PTK domain, may become potential antiproliferative agents and useful research tools. A preliminary report from our laboratory[13] has indeed demonstrated that PTK inhibitors which preferentially inhibit the epidermal growth factor receptor (EGFR) kinase block EGF-dependent cell proliferation. These blockers were found to inhibit poorly the PTK activity of the insulin receptor (InSR). Furthermore, we recently showed that PTK inhibitors which block insulin-dependent PTK activity of insulin receptor also inhibit insulin-induced lipogenesis and its antilipolytic effect in isolated rat adipocytes.[14] In this article, we report in detail on a large series of low molecular weight compounds which inhibit EGF receptor PTK activity at concentrations down to 3 orders of magnitude below the concentrations needed to inhibit insulin receptor PTK activity. We also showed that many of the potent EGF receptor kinase blockers inhibit the EGF-dependent proliferation of A431/clone 15 cells, as well as the autophosphorylation of the receptor.

Design, Synthesis, and Biological Evaluation of Inhibitors

Umezawa and his colleagues[15] isolated and characterized the compound erbstatin (Table IV, **71**) from the medium of an actinomycete. This compound inhibits EGF-receptor autophosphorylation in membranes of A431 epidermoid carcinoma cells with an $IC_{50} \approx 14\ \mu M$.[15] This observation led us to design PTK inhibitors based on the structure of erbstatin. As stressed by us earlier,[13] we followed three guidelines: (i) Selection of competitive compounds which compete with the *substrate subsite* of EGFR kinase and not with ATP. It was previously shown that the PTK inhibitors quercetin and genistein, which compete with ATP, also inhibit other protein kinases. This may be the reason why these compounds are cytotoxic.[16-18] (ii) Selection of compounds which preferentially inhibit EGF as compared to the homologous insulin receptor kinase (InsRK).[19] (iii) Selection of compounds soluble in water as well as in mildly hydrophobic solvents such as alcohols and DMSO. Such solubility properties may allow the compounds to traverse the cell membrane and therefore be tested on intact cells.

The compounds described in this publication are derived from benzylidenemalononitrile (BMN) nucleus. These compounds resemble the moieties tyrosine and erbstatin at the same time. The direction of the amide bond in erbstatin is inverted and dehydrated relative to the cyano group in BMN. The second cyano group in BMN was found to increase the biological activity of these compounds,[13] a finding which simplified our drug design and allowed the use of relatively simplified synthetic protocols.

Scheme I



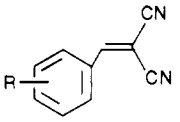
In vitro testing of PTK inhibitors has become simple because of the development of synthetic polymer substrates for PTKs. Different PTKs exhibit preference toward specific polymers. EGFR kinase prefers polyGAT [poly(Glu_6Ala_3Tyr)] whereas insulin receptor kinase (InsRK) prefers polyGT [polyGlu_4Tyr].[20] Therefore these two polymers were used as substrates for in vitro assay for EGFR kinase and InsRK. Since the polymers are of high molecular weight, they precipitate in trichloroacetic acid (TCA) such that a simple and inexpensive screening procedure could be set up. The human epidermoid carcinoma clonal cell line A431/clone 15, whose growth is stimulated 2.1–2.3-fold by EGF,[22] allowed us to test the PTK inhibitors on EGF-dependent proliferation.[13] Compounds were tested in vitro as inhibitors of EGFR-catalyzed poly(GAT) phosphorylation and as blockers of the EGF-dependent stimulation of A431/clone 15 cell proliferation. Selectivity of the PTK blockers was examined by measuring their inhibitory action on InsRK-catalyzed phosphorylation of poly(GAT) as compared to their effect on EGFR kinase (EGFRK).

Results and Discussion

Most of the benzylidenemalononitriles were prepared by straightforward Knoevenagel condensation of benzaldehydes or acetophenones with malononitriles, cyano-

(12) Chen, W. S.; Lazer, C. S.; Poenie, M.; Tsien, R. Y.; Gilo, G.-N.; Rosenfeld, M. G. *Nature* **1989**, *328*, 820.
(13) Yaish, P.; Gazit, A.; Gilon, C.; Levitzki, A. *Science* **1988**, *242*, 933.
(14) Shechter, Y.; Yaish, P.; Chorev, C.; Gilon, C.; Levitzki, A. *EMBO J.* **1989**, *8*, 1671.
(15) Umezawa, M.; Imoto, M.; Sawar, T.; Isshiki, K.; Matsuda, N.; Uchida, T.; Iinuma, H.; Hamada M.; Takeuchi, T. *J. Antibiot.* **1986**, *39* (1), 170.
(16) Glossmann, H.; Presek P.; Eigenlerodt, E. *Naunyn-Schmeideberg's Arch. Pharmacol.* **1981**, *317*, 100.
(17) Graziani, Y.; Erikson, E.; Erikson, R. L. *Eur. J. Biochem.* **1983**, *135*, 583.
(18) Akiyama, T.; Ishida, J.; Nakagawa, S.; Osaware, H.; Watanabe, S.; Itoh, N.; Shihuya, M.; Fukami, Y. *J. Biol. Chem.* **1987**, *262*, 5592.
(19) Riedel, H.; Dull, T. J.; Schlessinger, J.; Ulrich, A. *Nature* **1986**, *324*, 68.
(20) Braun, S.; Raymond W. E.; Racker, E. *J. Biol. Chem.* **1984**, *259*, 2051.
(21) Carson, B. B.; Stoughton, R. W. *J. Am. Chem. Soc.* **1928**, *50*, 2825.
(22) Bravo, R.; Burkhardt, J.; Curran, T.; Muller, R. *EMBO J.* **1985**, *4*, 1193.
(23) *Catechol Monoethers Morphine Alkaloids*; Holmes, H. L., Ed.; Suffield Ralston Publ.: Alberta, Canada, 1975; *Chem. Abstr.* **1976**, *84*, 163, 701.
(24) Horivuchi, F.; Fujimoto, K.; Ozaki, T.; Nishizawa, Y. *Agr. Biol. Chem.* **1971**, *35*, 2003.
(25) Weinberger, M. A.; Hlggie, R. M. *Can. J. Chem.* **1965**, *43*, 2585.
(26) Horner, L.; Klupfel, K. *Justus Liebigs Ann. Chem.* **1955**, *591*, 69.
(27) Mariella, R. P.; Bauer, J. M. *J. Org. Chem.* **1958**, *23*, 120.
(28) Rosemund, K. W.; Boem, T. *Justus Liebigs Ann. Chem.* **1924**, *437*, 125.
(29) Brunskill, J. S. A.; Vas, A. P. *Synth. Commun.* **1978**, *8*, 1.
(30) Astel, M. J.; Gergel, W. C. *J. Org. Chem.* **1956**, *21*, 493.
(31) Corey, E. J.; Fraenkel, G. *J. Am. Chem. Soc.* **1953**, *75*, 1168.
(32) Schonne, H. A.; Braye, E.; Bruylants, A. *Bull. Soc. Chim. Belg.* **1953**, *62*, 155; *Chem. Abstr.* **1954**, *48*, 9341.
(33) Fleury, J. P.; Libis, B. *Bull. Soc. Chim. Fr.* **1964**, 413.
(34) Patai, S.; Zabicky, J.; Israeli, Y. *J. Chem. Soc.* **1960**, 2038.
(35) Streith, J.; Cassal, J. M. *C. R. Seances Acad. Sci.* **1967**, 264C, 1307 (1967).
(36) Rodionov, V. M.; Dudinskaia, A. A.; Avramenko, V. G.; Suvarov, N. N. *J. Gen. Chem. USSR (Engl. Transl.)* **1958**, *28*, 2279.
(37) Westfahl, J. C.; Gresham, T. L. *J. Am. Chem. Soc.* **1954**, *76*, 1076.
(38) Fischer, E.; Nouri, O. *Chem. Ber.* **1917**, *50*, 611.

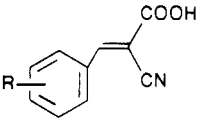
Table I. Data for 1–25



serial no.	R	method	% Yield	mp, °C	anal. or ref	IC_{50}, μm	K_I, μM
1	4-OCH_3	A	77	115	21	>1250[a]	>400[c]
2	3,5-$(OCH_3)_2$	A	77	85	23	>1250[a]	>400[c]
3	4-F	A	82	114	25	>1250[a]	>400[c]
4	3,5-(OCH_3)-4-OH	A	78	175	23	850[a]	300[c]
5	4-$NHCO(CH_2)_2COOH$	A	42	217	C, H, N	640[a]	240[c]
6	4-(*N*-morpholino)	E	85	190	29	>625[a]	>250[c]
7	3,4-OCH_2O-6-NO_2	B	80	104	C, H, N	560[a]	
8	4-OH	A	87	180	21	560	160 ± 10[d]
9	3,5-(*t*-Bu)-4-OH	A	50	135	24	460[a]	155[c]
10	3-OH-4-NO_2	A	78	134	C, H, N	450	150[c]
11	4-CH=CHCOOH	A	87	217	C, H, N	450	150[c]
12	4-COOH	B	85	190	23	430	143[c]
13	3-OH	A	94	150	26	375	123[c]
14	3,4-OCH_2O–	A	90	195	21	350[a]	117[c]
15	3-OH-4-OCH_3	A	94	250	21	>250[a]	*e*
16	3-OCH_3-4-OH	A	51	134	21	200	67[c]
17	3-OCH_3-4-OH-5-NO_2	A	64	140	C, H, N	160[a]	55[c]
18	3-OCH_3-4-OH-5-Br	A	72	170	27	153	50[c]
19	3-NO_2-4-OH	A	73	175	C, H, N	125	49[c]
20	3-OH-6-NO_2	B	85	210	C, H, N	96	24[c]
21	4-CHO	A[b]	85	142	C, H, N	60[a]	20[c]
22	3,5-$(OH)_2$	C	30	260	C, H, N	37	10[c]
23	3,4-$(OH)_2$	A	86	225	28	35	11 ± 0.1[d]
24	3,4-$(OH)_2$-5-OCH_3	D	46	235	C, H, N	6	2.2 ± 0.3[d]
25	3,4,5-$(OH)_3$	A	56	245	28	3	1

[a] The compound was dissolved in DMSO–EtOH–H_2O. Final concentration was 1% DMSO and 5% EtOH. [b] From the hydrolysis of the terephthaldehyde monoacetal product. IC_{50} determined at substrate concentration of $2K_m$. For cases in which K_I was computed from Dixon plots, the value is given. [c] Type of inhibition was analyzed according to Lineweaver–Burk and was found to be competitive toward the substrate. [d] Competitive inhibition studied in detail and demonstrated by using the analysis according to Dixon.[39] [e] Type of inhibition not analyzed.

Table II. Data for 26–36



serial no.	R	method	% yield[a]	mp, °C	anal. or ref	K_I, μM	IC_{50}, μM
26	H	F	62	192	30	>2500[b]	850[c]
27	4-OCH_3	F	86	218	30	2500	833[c]
28	4-F	F	88	170	C, H, N	2500	833[c]
29	4-$(CH_3)_2N$	F	66	226	32	1400	MC[f]
30	4-CH_3	F	76	208	31	1350[b]	*e*
31	4-NO_2	F	58	205	31	1300[b]	*e*
32	3-OCH_3-4-OH	F	71	223	30	800	325[c]
33	3,5-(OCH_3)-4-OH	F	54	225	C, H, N	700	264[c]
34	4-OH	F	86	257	30	308	165 ± 1.2[d]
35	4-CHO	F	36	202	C, H, N	140	47[c]
36	3,4-$(OH)_2$	F	93	240	28	70	25.0[d]

[a] Yield from the aldehyde. [b] See Table I footnote *a*. [c–e] As in Table I. [f] Mixed competitive.

acetamide, or malononitrile dimer (Scheme I).

The α-cyanocinnamic acids were prepared by condensation with *tert*-butyl cyano esters followed by mild hydrolysis with TFA. This route is more efficient and less prone to side products than direct condensation with cyanoacetic acid.

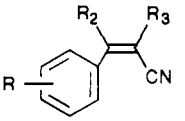
Several malononitriles were prepared by special methods: (1) nucleophilic substitutions of *p*-F-BMN, (2) reaction of anilines with (ethoxymethylene)malononitrile, and (3) reaction of malononitrile with diazonium salts or aroyl chlorides. These conditions are described in the Experimental Section.

The BMNs are easy to prepare and are stable compounds compared to erbstatine derivatives.

A large number of compounds were prepared and screened as inhibitors for EGFRK and InsRK and of inhibitors of EGF-dependent growth of A431/clone 15 cells. Tables I–IV summarize the properties of the compounds and their EGFRK-inhibitory activity. The IC_{50} values were determined under the standard in vitro assay conditions described in the Experimental Section, which employ a substrate concentration equal to 2 times the K_m. This allows an estimation of the inhibition constant, assuming competitive inhibition. For a large number of compounds we have analyzed in detail the inhibitory properties of the compounds as shown in Tables I–IV.

Nature of Inhibition. We have examined the nature of EGFRK inhibition and found that many of the com-

Table III. Data for 37–53



serial no.	R_1	R_2	R_3	method	% yield	mp, °C	anal. or ref	IC_{50}, μM	K_I, μM
37	4-NH_2	CH_3	CN	A	18	190	23	>1250[a]	*e*
38	3-OCH_3-4-OH	CH_3	COOH	F	20	182	C, H, N	1100[a]	341[c]
39	4-OH	H	C(=O)NH_2	A	46	250	34	800	*e*
40	4-OH	H	C(=O)$NHCH_2CN$	A	42	238	C, H, N	625	360[c]
41	4-$NHC(=O)(CH_2)_2COOH$	CH_3	CN	A	26	180	C, H, N	500	NM[g]
42	4-OCH_3	OH	CN	G	13	185	33	450	*e*
43	4-OH	OH	CN	G	20	195	C, H, N	230	*e*
44	3,4,5-$(OH)_3$	OH	CN	G	18	oil	C, H, N	13.5	4.5[c]
45	3,5-(Bu)-4-OH	H	C(=O)NH_2	A	66	200	24	55[a]	*e*
46	3,4-$(OH)_2$	H	C(=O)NH_2	A	70	247	28	10	3.5[d]
47	3,4-$(OH)_2$	H	C(=S)NH_2	A	41	213	C, H, N, S	2.4	0.85[c]
48	4-OH	H	$C(NH_2)$=$C(CN)_2$	A	70	225	C, H, N	0.125	MC[f]
49	3-NO_2-4-OH	H	$C(NH_2)$=$C(CN)_2$	A	86	219	C, H, N	60	
50	3,4-$(OH)_2$	H	$C(NH_2)$=$C(CN)_2$	A	53	235	C, H, N	2.5	MC[f]
51	3,4,5-$(OH)_3$	H	$C(NH_2)$=$C(CN)_2$	A	87	275	C, H, N	0.8	MC[f]
52	3,4-(OH)-5-OCH_3	H	$C(NH_2)$=$C(CN)_2$	B	90	225	C, H, N	1.2	MC[f]
53	3,4-$(OH)_2$-5-Br	H	$C(NH_2)$=$C(CN)_2$	B	77	241	C, H, N	0.5	*e*

[a,c,d,e] As in Table I. [f] Mixed competitive. [g] Non-Michaelian.

pounds are competitive with the exogenous substrate polyGAT. Detailed kinetic analysis according to the Dixon method[39] for a large number of compounds revealed that many are competitive whereas some are mixed-competitive (Figures 1 and 2 and Tables I–IV). In this article we have concentrated on the competitive inhibitors which reveal minimal cytotoxicity up to concentrations of 100 μM or above.[13] In a separate study the detailed behavior of the mixed-competitive inhibitors and their biological effects will be presented. The pattern emerging is that the mixed-competitive and noncompetitive inhibitors possess an extended side chain (Tables III and IV).

Specificity of the Compounds. A selection of the compounds were tested as inhibitors of InsRK. All the compounds tested were found to be poor inhibitors of InsRK in spite of its high degree of homology to EGFRK in the PTK domain.[40] Although the compounds found to be competitive inhibitors of EGFRK were also found to be competitive inhibitors of InsRK (Figure 2), their affinity for the latter was down to 3 orders of magnitude lower than that for EGFRK[13] (Figure 2). Still, the fact that these tyrphostins can inhibit competitively InsRK can form the basis for the selective inhibitors for the InsRK.

Structure–Activity Relationship. The substructure responsible for the potency of BMNs as PTK inhibitors appears to be *cis*-polyhydroxycinnamonitrile. Thus cinnamonitrile **64** is inactive and saturation of the double bond in **8** to give **63** abolished activity. Electron-withdrawing groups (as in **10–12**, **17**, **31**) or electron-donating groups (as in **1**, **29**, **30**) make relatively little difference. Although nitro or bromo groups seem to increase the potency and a methoxy group seems to decrease it, their effect may be due to differences in solubility (vide infra). A strong increase in potencies is seen when hydroxyl groups are added, with 10–15-fold increase in potency for one OH added and a 3–5-fold increase for the other OH (IC_{50} (μM) for di-CN compounds: **8**, 560; **23**, 35; **25**, 3. IC_{50} (μM) for compounds with CN, COOH: **34**, 300; **36**, 70. IC_{50} (μM) for compounds with CN, amide: **39**, 800; **46**, 10. IC_{50} (μM) for compounds with CN, NH_2C=$C(CN)_2$: **48**, 125; **50**, 2.5; **51**, 0.8). When trans substitution is added to the *cis*-cinnamonitrile nucleus, an improvement is obtained: COOH < CN < C(=O)NH_2 < C(=S)NH_2 ≈ $C(NH_2)$=$C(CN)_2$ (for example, IC_{50} (μM): **36** (COOH), 70; **23** (CN), 35; **46** (amide), 10; **47** (thio amide), 2.4; **50**, 2.5).

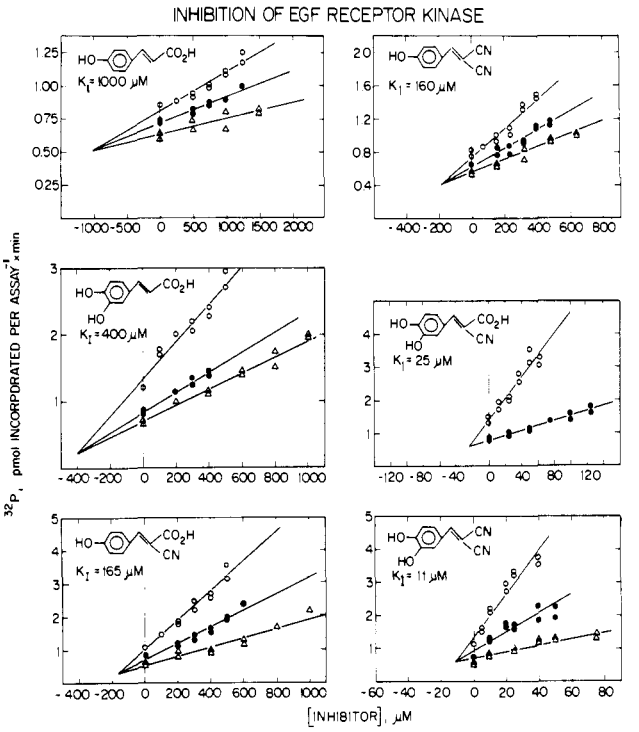
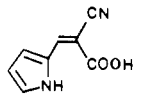
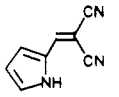
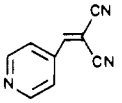
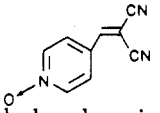
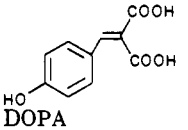
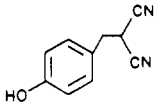
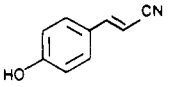
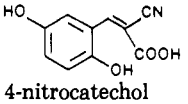
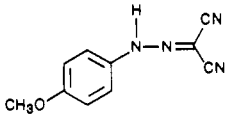
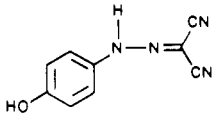
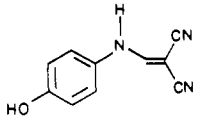
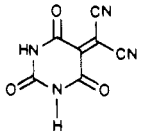
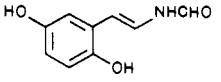


Figure 1. BMN derivatives are competitive inhibitors of the EGFRK substrate (tyrosine) site. The inhibitory effect of BMN derivatives as competitive inhibitors of EGFRK were examined by using the method of Dixon[39] (see the text). The substrate (polyGAT) concentrations used were equal to 0.5, 1, and 2 times the K_m values. Other experimental details are given in the text.

The SAR trends described in this article differ in the following aspects from the SAR described for erbstatin and its derivatives. (a) In the BMN inhibitors, a *cis*-cyano group is essential for high potency whereas in erbstatin the formamide group is trans. (b) Progressive addition of hydroxy substituents on the phenolic ring is more effective

(39) Dixon, M. *Biochem. J.* **1953**, *55*, 170.
(40) Yarden, Y.; Ulrich, A. *Annu. Rev. Biochem.* **1988**, *57*, 443.

Table IV. Data for 54–71

serial no.	formula	method	% yield	mp, °C	anal. or ref	IC_{50}, μM	K_I, μM
54		F	47	240	C, H, N	1360	–[f]
55		A	87	128	35	260[e]	–
56		A	20	oil	C, H, N	250	NM[i]
57		A	39	198	C, H, N	1300	–
58	hydroxybenzoic acid	*a*				not active	
59	*p*-hydroxycinnamic acid	*a*				3000	1000[g]
60	3,4-dihydroxycinnamic acid	*a*				1200	400[h]
61		F[b]	51	210	36	1500	–[f]
62	DOPA	*a*				900	–[f]
63		*c*	59	oil	37	6500	–[f]
64		*d*	20	128	38	2400	–[f]
65		A	83	285	C, H, N	75	24[f]
66	4-nitrocatechol	*a*				not active	
67		H	23	135	C, H, N	600	MC[j]
68		H	10	95	C, H, N	225	–[f]
69		I	89	245	C, H, N	350	–[f]
70		A	26	235	C, H, N	1400	–[f]
71		ref 15				14[k]	

[a] From Aldrich Co. [b] From di-*tert*-butyl malonate by TFA hydrolysis. [c] From **8** with $NaBH_4$. [d] From commerical *p*-methoxycinnamonitrile with BBr_3. [e] See Table I. [f] Type of inhibition not analyzed. [g] Type of inhibition analyzed according to Lineweaver–Burk. [h] Type of inhibition analyzed according to Dixon.[39] [i] Non-Michaelian. [j] Mixed competitive. [k] Autophosphorylation; see ref 15.

in the BMN analogues than in the erbstatin analogues.[41] (c) The *p*-hydroxycinnamic acid is a very weak inhibitor (K_I = 1600 μM) and *p*-hydroxybenzoic acid is inactive when measured by the in vitro assay described in this article, whereas Ogawara et al.[42] reported IC_{50} = 61 μM for *p*-hydroxybenzoic acid. We also found that caffeic acid (3,4-dihydroxycinnamic acid) has an IC_{50} value of 1200 μM, using a substrate concentration equal to 2 times the K_m

(41) Isshiki, K.; Imoto, M.; Takeuchi, T.; Umezawa, H.; Tsuchida, T.; Yoshioka, T.; Tatsuta, K. *J. Antibiot.* **1987**, *40*, 1207, 1209.

(42) Ogawara, H.; Akiyama, T.; Ishida, J.; Watanabe, S. I.; Suzuki, K. I. *J. Antibiot.* **1986**, *39*, 606.

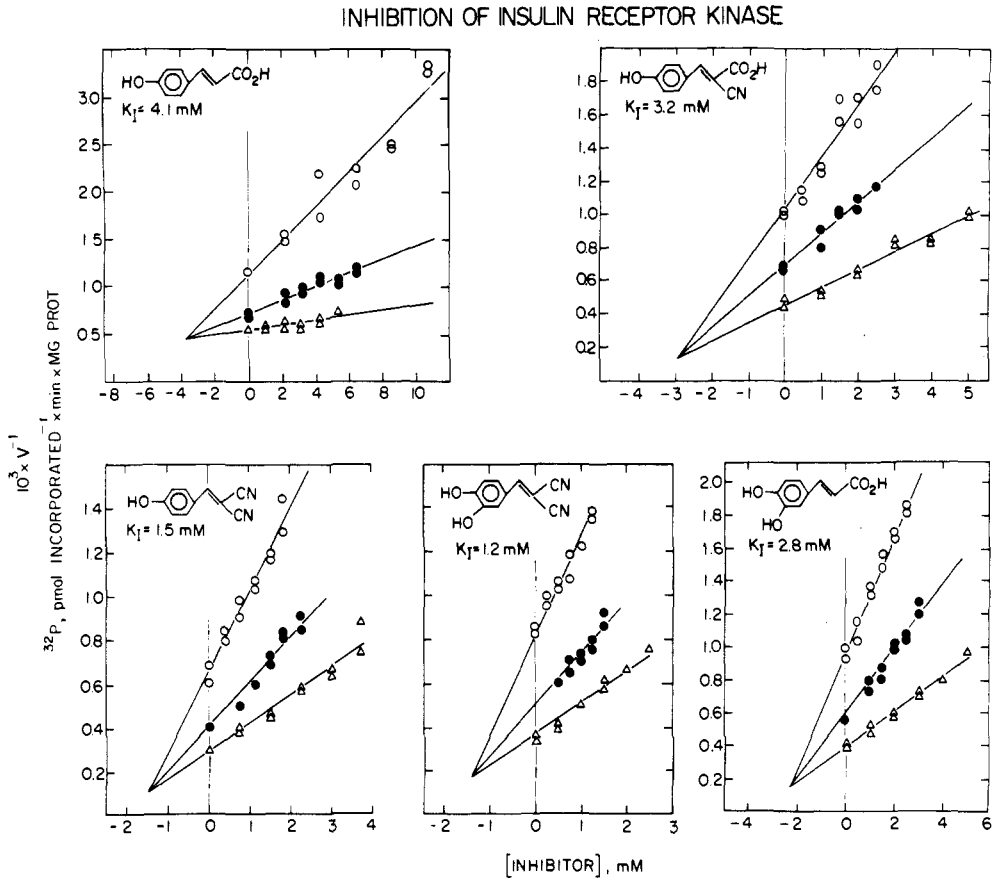


Figure 2. Inhibition of insulin-receptor kinase by BMN derivatives. Experimental details are given in the text. Analysis was carried out by using the Dixon[39] method as depicted.

value. Our value is much higher than that reported for the similar compound 2,5-dihydroxycinnamic acid[41] (4.4 μM). (d) We found that hydrophobic substituents on the phenolic ring reduce the efficacy. On the other hand, Shiraishi et al.[43,44] reported highly potent inhibitors which have hydrophobic substitutions on the phenolic ring. (e) Four compounds reported by Shiraishi et al.[43] exhibit potency in a very narrow range (IC_{50} = 0.44–0.85 μM) despite the fact that they have very different substitutions on the phenolic ring. In contrast to Shiraishi et al., we find that the inhibitory potency of the BMN series strongly depends on the nature of the substitutions and therefore exhibit a wide range of affinities (see the tables).

Inhibition of EGFR Autophosphorylation. The autophosphorylation of EGF receptor as well as of other growth-factor receptors, is believed to be the first step in signal transduction and a prerequisite for the phosphorylation of exogenous substrates by the receptor.[2,45–47] Figure 3 shows that EGF-receptor autophosphorylation is inhibited effectively by the competitive inhibitors which inhibit the EGF receptor catalyzed phosphorylation of exogenous substrate. The inhibitors are seemingly less effective (~10 fold) as blockers of EGF-receptor autophosphorylation than as blockers of exogenous substrate phosphorylation. This behavior is most probably due to the fact that the autophosphorylation reaction occurs *within* the EGF-receptor dimer[48] such that the inhibitor must compete against a very high local substrate concentration. Because of the "proximity effect", the substrate concentration can be estimated to be in the high molar range.[13,49] Accurate inhibition constants, however, cannot be calculated since the actual concentration of the autophosphorylated sites cannot be measured. These considerations strongly argue that the PTK inhibitors block autophosphorylation extremely effectively.

Conclusions and Significance. In this study and in our previous report,[13] we have demonstrated that it is feasible to prepare selective and nontoxic protein tyrosine kinase inhibitors. Thus, the ability to inhibit specifically receptor kinase in vitro and EGF-dependent cell proliferation[13] strongly suggests that proliferative conditions in which EGF and its receptor are implicated may be good candidates for treatment by tyrophostins of the family described in this and our previous[13] study. Such a pathological conditions is psoriasis, which is typified by the overexpression of transforming growth factor α, which leads to enhanced activity of the EGF receptor and therefore to proliferation of keratinocytes.[50]

In principle, it should be feasible to synthesize selective tyrphostins for each one of the known protein tyrosine kinases involved in cell proliferation and transformation. Our initial success to demonstrate a 10^2–10^3-fold difference

(43) Shiraishi, T.; Domoto, T.; Imai, N.; Shimada, Y.; Watanabe, K. *Biochem. Biophys. Res. Commun.* **1987**, *147*, 322.
(44) Shiraishi, T.; Kameyama, K.; Imai, N.; Domoto, T.; Katsumi, I.; Watanabe, K. *Chem. Pharm. Bull.* **1988**, *36*, 974.
(45) Yarden, Y.; Schreiber, A. B.; Schlessinger, J. *J. Cell Biol.* **1982**, *92*, 687.
(46) Westermark, B.; Wasterspm, A.; Heldin, C.-H. *Nature* **1982**, *307*, 521.
(47) Kasuga, M.; Karlsson, F. A.; Kahn, C. R. *Science* **1982**, *215*, 185.
(48) Yarden, Y.; Schlessinger, J. *Biochemistry* **1987**, *26*, 1434.
(49) Lehninger, A. In *Principles of Biochemistry*; Worth Publishers, Inc.: New York, 1982; p 225.
(50) Elden, J. T.; Fisher, G. J.; Lindquist, P. B.; Bennett, G. L.; Pitelkow, M. R.; Coffey, R. J., Jr.; Ellingsworth, L.; Derynck, R.; Voorhees, J. J. *Science* **1989**, *243*, 811.

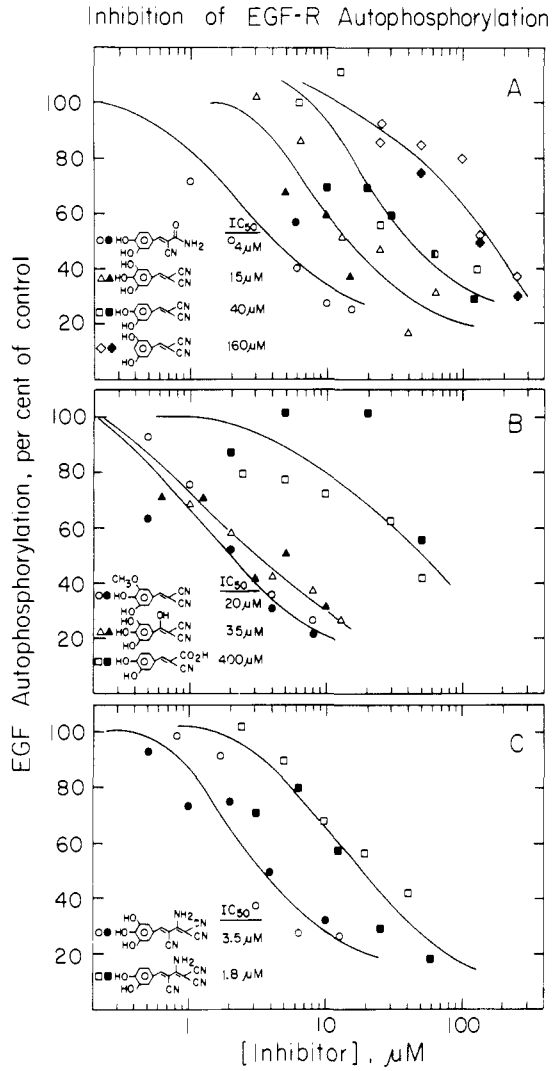


Figure 3. Inhibition of EGFRK autophosphorylation by BMN derivatives. EGFRK was incubated with the indicated concentrations of inhibitors as described in the experimental section. Open and filled symbols are duplicate experiments.

in the apparent affinity of a large series of tyrphostins to EGF receptor as compared to insulin receptor is a first step toward this goal.

Experimental Section

Materials and Methods. All aldehydes and ketones used were purchased from Aldrich. Malononitrile, cyanoacetamide, and thiocyanoacetamide were from Aldrich. 2-Amino-1,1,3-tricyanopropene and *tert*-butyl cyanoacetic ester were purchased from Fluka. Melting points are uncorrected. ^1H and ^{13}C NMR spectra were recorded on a Bruker WP-200 pulsed FT spectrometer. Mass spectra were recorded with a MAT 311 instrument. Aprotinin, benzamidine, *p*-methylbenzenesulfonyl fluoride (PMSF), soybean trypsin inhibitor (SBTI), leupeptin, *N*-acetyl-D-glucosamine, polyGAT, poly(Glu_4Tyr), and Triton X-100, were all from Sigma. EGF was obtained from International Diagnostic Laboratories (IDL), Jerusalem, insulin was from Elly Lilly, and wheat germ agglutininn (WGA) agarose was from Bio-Makor, Rehovoth. All other chemicals were of the highest purity available. All solutions were prepared with Corning-distilled, deionized water.

Workup means adding water to the reaction mixture and extracting it with $CHCl_3$ (or ethyl acetate for polar compounds) and washing the organic phase to neutrality, drying on $MgSO_4$, filtering, and evaporating the phase to dryness.

Synthetic Methods. Typical procedures for each class of compounds are given with one detailed example. All compounds (Tables I–IV) were fully characterized spectroscopically (^1H NMR, MS, and ^{13}C NMR for several compounds). Chemical shifts are in ppm relative to a TMS internal standard. Combustion analyses were within ±0.4% of the theoretical value.

(3,4-Dihydroxybenzylidene)malononitrile (23) (Method A). To 11 g (80 mM) of 3,4-dihydroxybenzaldehyde and 5.5 g (83 mM) of malononitrile in 60 mL of ethanol were added 10 drops of piperidine and the reaction refluxed for 0.5 h. Water was added and the solid was filtered and dried to give 12.7 g (86% yield) of yellow solid: mp 225 °C (lit.[28] mp 225 °C); MS *m/e* 186 (M^+, 100), 159 (M − HCN, 10), 158 (M − CO, 59), 157 (M − HCO, 26), 130 (M − 2 CO, 90), 129 (54), 113 (21), 103 (M − 2 CO − HCN, 74); NMR (acetone-d_6) δ 7.03 (1 H, d, $J_{5,6}$ = 8.4 Hz, H 5), 7.41 (1 H, dd, $J_{5,6}$ = 8.4 Hz, $J_{2,6}$ = 2.1 Hz), 7.68 (1 H, d, $J_{2,6}$ = 2.1 Hz), 8.04 (1 H, s, vinylic proton); ^{13}C NMR (acetone-d_6) δ 77.4 (C_α), 114.7, 115.6 (CN), 116.9 (C_5), 125.0 (C_1), 130.0 (C_2), 146.6 (C_6), 153.3 (C_4), 160.7 (C_β).

The same procedure was used for the preparation of **39**, **45–47**, and **48–53** except that cyanoacetamide, thiocyanoacetamide, and malononitrile dimer (2-amino-1,1,3-tricyanopropene) were used respectively.

[3,4-(Methylenedioxy)-6-nitrobenzylidene]malononitrile (7) (Method B). 3,4-(Methylenedioxy)-6-nitrobenzaldehyde (1 g, 5.1 mM), 0.4 g (6 mM) of malononitrile, and 0.2 g of β-alanine in 30 mL of ethanol were stirred for 16 h at room temperature. H_2O (50 mL) was added. Filtering gave 1 g (80% yield) of bright yellow solid: mp 104 °C; NMR (acetone-d_6) δ 6.42 (2 H, s, methylenedioxy), 7.45 (1 H, s, H_2), 7.82 (1 H, s, H_5), 8.70 (1 H, s, vinylic proton). Anal. ($C_{11}H_5N_3O_4$) C, H, N.

(3,5-Dihydroxybenzylidene)malononitrile (22) (Method C). To 0.8 g (3.7 mM) of (3,5-dimethoxybenzylidene)malononitrile (**2**) in 30 mL of CH_2Cl_2 was added 1.3 mL (13 mM) of BBr_3. After stirring for 4 h at room temperature, 100 mL of 3 N HCl was added, and the solution was extracted with 3 × 70 mL of ethyl acetate. Workup gave 0.2 g of yellow-green solid: 29% yield; mp 260 °C; NMR (acetone-d_6) δ 6.24 (1 H, t, J = 2.0 Hz, H_4), 6.74 (2 H, d, J = 2.0 Hz, $H_{2,6}$), 7.70 (1 H, s, vinylic proton). Anal. ($C_{10}H_6N_2O_2$) C, H, N.

(3,4-Dihydroxy-5-methoxybenzylidene)malononitrile (24) (Method D). To 0.46 g (2 mM) of **4** in 30 mL of trichloroethylene were added 0.3 g (1.1 mM) of $AlCl_3$ and 0.8 mL of pyridine. After 3 h of reflux and cooling, the solvent was decanted, and the remaining sticky solid was treated with 100 mL of 6 N HCl and extracted with ethyl acetate (2 × 50 mL). Workup and chromatography on silica gel gave 0.2 g (46% yield) of yellow solid: mp 235 °C; NMR (acetone-d_6) δ 3.88 (3 H, s, OCH_3), 7.30 (1 H, d, J = 2.0 Hz, H_2), 7.32 (1 H, d, J = 2.0 Hz, H_6), 8.03 (1 H, s, vinylic proton). Anal. ($C_{11}H_8N_2O_3$) C, H, N.

(4-Morpholinobenzylidene)malononitrile (6) (Method E). To 0.85 g (5 mM) of **3** in 40 mL of ethanol was added 1.1 g (12.6 mM) of morpholine. After 2 h of reflux, the reaction was cooled and allowed to stand for 1 h. Filtering gave a yellow solid which was recrystallized from ethanol to give 0.82 g (85% yield) of solid: mp 188 °C (lit.[29] mp 195 °C); NMR ($CDCl_3$) δ 3.65 (8 H, m), 7.10, 7.94 (4 H, AB q, J_{AB} = 9.3 Hz), 7.93 (1 H, s, vinylic proton).

***p*-Nitro-α-cyanocinnamic Acid (31) (Method F). (a).** To 1.5 g (10 mM) of *p*-nitrobenzaldehyde and 2 g (14 mM) of *tert*-butyl cyano acetic ester in 30 mL of ethanol was added 10 drops of piperidine. After 0.5 h of reflux, 100 mL of ice–water was added and the precipitate formed was filtered and recrystallized from ethanol–water to give 2.3 g (64%) of a light-yellow solid: mp 126 °C; NMR (acetone-d_6) δ 1.59 (9 H, s, *tert*-butyl), 8.29, 8.43 (4 H, AB q, J_{AB} = 8.6 Hz), 8.42 (1 H, s, vinylic proton). Anal. ($C_{14}H_{14}N_2O_4$) C, H, N.

(b). The cyano ester from (a) (2.1 g) was dissolved in 10 mL of trifluoroacetic acid. After 30 min of stirring at room temperature, a solid precipitated. TFA was evaporated in vacuo, the solid was triturated with water, filtered, and dried to give 1.5 g of yellow-orange solid: 90% yield; mp 205 °C (lit.[31] 210 °C); NMR (acetone-d_6) δ 6.0 (1 H, br s, COOH), 8.32, 8.45 (4 H, AB q, J_{AB} = 9.0 Hz), 8.49 (1 H, s, vinylic proton).

(3,4,5,β-Tetrahydroxybenzylidene)malononitrile (44) (Method G). (a). To 30 g (0.17 mol) of gallic acid in 120 mL of Ac_2O was added 10 drops of concentrated H_2SO_4. The clear solution was heated at 80 °C for 10 min, cooled, and decomposed with caution by adding it to 300 mL of ice–water. After 2 h at room temperature, the white solid was filtered and dried to yield

40 g (77% yield) of triacetyl gallic acid: mp 163 °C; NMR (acetone-d_6) δ 2.31 (6 H, s, acetyl at 3, 5), 2.33 (3 H, s, acetyl at 4), 7.80 (2 H, s, $H_{2,6}$).

(**b**). To 7 g (23.6 mM) of triacetylgallic acid was added 5 mL (70 mM) of $SOCl_2$ in 50 mL of C_6H_6. Reflux (1.5 h) and evaporation to dryness gave a white solid. This acid chloride was dissolved in 50 mL of CH_2Cl_2 and added dropwise to 2 g (30 mM) of malononitrile and 4 mL of Et_3N in 100 mL of CH_2Cl_2. After 3.5 h of stirring at room temperature, 100 mL of 2 N HCl was added and the reaction was extracted with 3 × 50 mL of $CHCl_3$ and worked up to yield a red sticky oil: 5.5 g; NMR (acetone-d_6) δ 2.28 (6 H, s), 2.30 (3 H, s), 7.96 (2 H, s). This oil was dissolved in 30 mL of ethanol and 40 mL of water. NaOH (0.5 g) was added and the solution was heated at 80 °C for 2 min and then acidified with HCl to pH = 4 and extracted with 3 × 50 mL of $CHCl_3$. Workup gave a brown oil, which was flash-chromatographed on silica gel to yield 1 g of oil: NMR (acetone-d_6) δ 7.11 (s). Anal. ($C_{10}H_6N_2O_4$) C, H, N.

[(*p*-Methoxyphenyl)hydrazono]malononitrile (67) (Method H). *p*-Anisidine (12.2 g, 0.1 M) was dissolved in 30 mL of concentrated HCl and 20 mL of water cooled in ice and then 7 g of $NaNO_2$ in 50 mL of water was added in portions. A mixture of 6.6 g (0.1 M) of malononitrile, 20 g of NaAc, 15 mL of ethanol, and 50 mL of water was prepared separately and cooled in ice. The diazonium salt solution was added slowly to the second solution, with ice cooling. The cooled reaction was stirred for 0.5 h and filtered to give a yellow-green solid: 4.6 g; 23% yield; mp 135 °C; NMR ($CDCl_3$) δ 3.83 (3 H, s, OCH_3), 6.95, 7.25 (4 H, AB q, J_{AB} = 9.0 Hz), 9.95 (1 H, br s, NH). Anal. ($C_{10}H_8N_4O$) C, H, N.

[(*p*-Hydroxyanilino)methylene]malononitrile (69) (Method I). *p*-Aminophenol (1 g, 9 mM) and 0.85 g (7 mM) of (ethoxymethylene)malononitrile in 40 mL of acetonitrile were refluxed for 1.5 h. Evaporation to dryness gave a yellow solid which recrystallized from ethanol to give 1.15 g (89% yield) of a white solid: mp 245 °C dec; NMR (acetone-d_6) δ 6.88, 7.32 (4 H, AB q, J_{AB} = 9.0 Hz), 8.23 (1 H, s, vinylic proton). Anal. ($C_{10}H_7N_3O$) C, H, N.

Biochemical Methods. EGF-Receptor Purification. EGF-receptor purification was based on the procedure of Yarden and Schlessinger.[48] A431 cells were grown in 80 cm^2 bottles to confluency (2 × 10^7 cells per bottle). The cells were washed twice with PBS and were harvested with PBS containing 1.0 mM EDTA (1 h at 37 °C), and centrifuged at 600*g* for 10 min. The cells were solubilized in 1 mL per 2 × 10^7 cells of cold solubilization buffer (50 mM Hepes buffer, pH 7.6, 1% Triton X-100, 150 mM NaCl, 5 mM EGTA, 1 mM PMSF, 50 μg/mL aprotinin, 25 mM benzamidine, 5 μg/mL leupeptin, and 10 μg/mL soybean trypsin inhibitor) for 20 min at 4 °C. After centrifugation at 100000*g* for 30 min, the supernatant was loaded onto a WGA-agarose column (100 μL of packed resin per 2 × 10^7 cells) and shaken for 2 h at 4 °C. The unabsorbed material was removed and the resin was washed twice with HTN buffer (50 mM Hepes, pH 7.6, 0.1% Triton X-100, 150 mM NaCl), twice with HTN buffer containing 1 M NaCl, and twice with HTNG buffer (50 mM Hepes, pH 7.6, 0.1% Triton X-100, 150 mM NaCl, and 10% glycerol). The EGF receptor was eluted batchwise with HTNG buffer containing 0.5 M *N*-acetyl-D-glucosamine (200 μL per 2 × 10^7 cells). The eluted material was stored in aliquots at −70 °C and diluted before use with TMTNG buffer (50 mM Tris–Mes buffer, pH 7.6, 0.1% Triton X-100, 150 mM NaCl, 10% glycerol).

EGFR Kinase Catalyzed Phosphorylation of Poly(GAT) and Its Inhibition. WGA-purified EGFR (0.25 μg/assay) was preactivated with EGF (0.85 μM) in 50 mM Tris–Mes buffer, pH 7.6 for 20 min at 4 °C. The assay was initiated by addition of a mixture which contained $Mg(Ac)_2$ (60 mM), [γ-^{32}P]ATP (125 μM, 2–5 μCi/assay), poly(GAT) (0.0625 mg/mL, 0.125 mg/mL, 0.25 mg/mL), and six concentrations of inhibitor in duplicates. The temperature of the assay was 22 °C and the production of phosphorylated copolymer was found to be linear up to 20 min (data not shown, ref 51). The PTK inhibitors tested were solubilized in water or a mixture of ethanol and water such that the final concentration of ethanol did not exceed 4% in the assay. Up to 4% ethanol in the assay had no effect on the EGFR kinase activity (data not shown, ref 52). The final concentration of EGF in the assay was 300 nM in a final volume of 40 μL. After 5, 10, or 20 min, aliquots of 25 μL were applied onto Whatman 3-mm paper cuttings, which were then soaked in cold 10% TCA containing 0.01 M sodium pyrophosphate. After being washed overnight at 4 °C, the paper cuttings were dried and counted, measuring ^{32}P Cerenkov radiation. Concentration dependence on poly(GAT) was Michaelian with a K_m = 0.076 ± 0.007 mg/mL or 0.069 ± 0.007 mM if calculated per Glu_6Ala_3Tyr(GAT) unit (Figure 4). The EGF dose response for the poly(GAT) phosphorylation is shown in Figure 5. The K_m for ATP in the assay was found to 2.9 μM, similar to published values.[52]

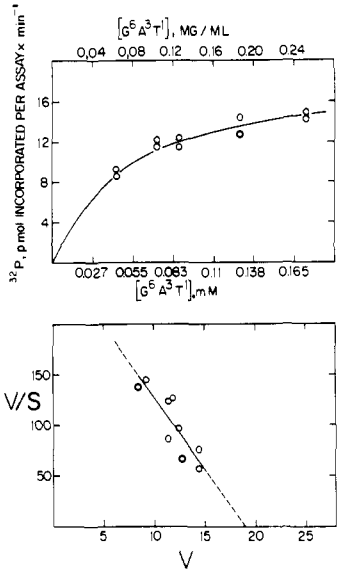


Figure 4. The concentration dependence of poly(GAT) phosphorylation by EGFRK. The phosphorylation of poly(GAT) was conducted as described in the Experimental Section. The concentration of the polymer was varied up to 0.25 mg/mL. The kinetic pattern was found to be Michaelian with K_m = 0.065 mM calculated for the Glu_6Ala_3Tyr unit.

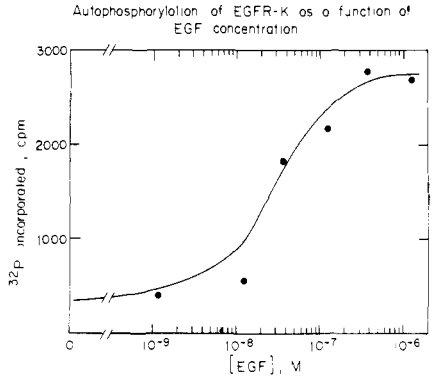


Figure 5. The EGF concentration dependence of poly(GAT) phosphorylation by EGFRK.

Time Dependence of EGF-Receptor Autophosphorylation. WGA-purified EGF receptor from A431 cells (0.5 μg/assay) was activated with EGF (800 nM) for 20 min at 4 °C. The reaction was initiated by the addition of $Mg(Ac)_2$ (60 mM), Tris–Mes buffer, pH 7.6 (50 mM), and [^{32}P]ATP (20 μM, 5 μCi/assay). The reaction was conducted at either 4 or 15 °C and terminated by addition of sodium dodecyl sulfate (SDS) sample buffer (10% glycerol, 50 mM Tris, pH 6.8, 5% β-mercaptoethanol, and 3% SDS). The samples were run on a 8% SDS polyacrylamide gel (SDS-PAGE) (prepared from 30% acrylamide and 0.8% bis-(acrylamide) and contained 0.375 M Tris, pH 8.8, 0.1% SDS,

(51) Yaish, P. Ph.D. Thesis, The Hebrew University of Jerusalem 1989.

(52) Cuatrecasas, P. *Proc. Natl. Acad. Sci. U.S.A.* **1972**, *69*, 318.

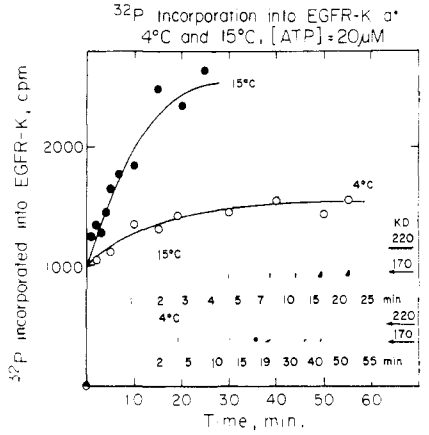


Figure 6. Kinetics of EGF receptor kinase autophosphorylation. The kinetic results at two temperatures are depicted. Experimental details are given in the text.

0.05% TEMED, and 0.46% ammonium persulfate). The gel was dried and autoradiography was perfromed with Agfa Curix RP2 X-ray film. The relevant radioactive bands were cut and counted in the Cerenkov mode. The fast phase of autophosphorylation continued for another 10 min (data not shown). The extent of phosphorylation completed in the first 10 s at 15 °C comprises $^1/_3$ of the total autophosphorylation signal and probably reflects the phosphorylation of the first site on the receptor (Figure 6). The 10-s interval was therefore chosen for use in subsequent autophosphorylation experiments.

ATP and EGF Dependence of Autophosphorylation. WGA-purified EGF receptor from A431 cells (0.5 μg/assay) was activated with EGF (0.85 μM) for 20 min at 4 °C. The assay was performed at 15 °C and initiated by addition of $Mg(Ac)_2$ (60 mM), Tris–Mes buffer, pH 7.6 (50 mM), [^{32}P]ATP (carrier free, 5 μCi/assay), and increasing concentrations of nonradioactive ATP. The assay was terminated after 10 s by addition of SDS sample buffer (see above). The samples were run on a 6% SDS polyacrylamide gel (see above). The gel was dried and autoradiographed as described above (Figure 6). The relevant radioactive bands were cut and counted in the Cerenkov mode. The K_m for ATP determined in this fashion was found to be 7.2 μM[51] (data not shown). With use of the 10-s assay protocol, the EGF concentration dependence of EGFRK autophosphorylation was determined and found to be similar to that shown in Figure 5 (data not shown).[51]

Inhibition of EGFR Autophosphorylation. WGA-purified EGF receptor from A431 cells (0.5 μg/assay) was activated with EGF (0.85 μM) for 20 min at 4 °C. The assay was performed at 15 °C and initiated by addition of $Mg(Ac)_2$ (60 mM), Tris–Mes buffer, pH 7.6 (50 mM), and [γ-^{32}P]ATP (10 μM, 5 μCi/assay) and various inhibitors at increasing concentrations. The assay was terminated after 10 s by the addition of SDS-PAGE sample buffer.

Inhibition of Copoly(Glu$_4$Tyr) Phosphorylation by Insulin-Receptor Kinase (InsRK). Rat liver membranes were prepared from the livers of 6-week-old rats as described by Cuatrecasas.[52] WGA-purified insulin receptor was prepared according to Zick et al.[53] WGA-purified rat liver InsRK (1.25 μg) was preincubated with or without 330 nM insulin in 50 mM Tris–Mes buffer, pH 7.6, for 30 min at 22 °C. The assay was performed at 22 °C and initiated by addition of a mixture which contained $Mg(Ac)_2$ (60 mM), $NaVO_3$ (40 μM), [γ-^{32}P]ATP (125 μM, 3–5 μCi/assay), and poly(GT) [poly(Glu$_4$Tyr)] at three concentrations: whenever an inhibitor was tested, it was added at the proper concentration as indicated. The concentrations referred to are the final concentration in the assay. The final concentration of insulin in the assay was 125 nM. The total volume of the assay was 40 μL. After 20 min, aliquots of 30 μL were applied on Whatman 3-mm paper and were soaked in cold 10% TCA, containing 0.01 M sodium pyrophosphate. After being washed overnight, the papers were dried and counted, measuring Cerenkov radiation. The InsRK-catalyzed phosphorylation of poly(GT) obeys Michaelis–Menten kinetics[14] and the inhibitors are purely competitive as was observed from the well-behaved Dixon[39] plots[14] (data not shown).

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(53) Zick, Y.; Kasuga, M.; Kahn, R.; Roth, J. *J. Biol. Chem.* **1983**, *258*, 75.

Synthesis and Investigation of Effects of 2-[4-[[(Arylamino)carbonyl]amino]phenoxy]-2-methylpropionic Acids on the Affinity of Hemoglobin for Oxygen: Structure–Activity Relationships

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A series of 2-[4-[[[(substituted-phenyl)amino]carbonyl]amino]phenoxy]-2-methylpropionic acids and other substituted phenoxyacetic acids were synthesized and tested for their ability to reduce the affinity of hemoglobin for oxygen. 2-[4-[[[(3,4,5-trichlorophenyl)amino]carbonyl]amino]phenoxy]-2-methylpropionic acid was found to be the most potent compound known. Structure–activity relationships of the compounds synthesized are discussed.

Perutz and Poyart in 1983 reported that the antihyperlipidemic agent bezafibrate lowered the affinity of hemoglobin for oxygen.[1] We have reported that 2-[4-[[[(3,4-dichlorophenyl)amino]carbonyl]amino]phenoxy]-2-methylpropionic acid (**8g4**, LR-16), a newly synthesized compound related to clofibric acid, has an even higher affinity for hemoglobin and causes a larger shift of the hemoglobin–oxygen-dissociation curve to the right.[2] X-ray crystallography demonstrated that the site in the central cavity of hemoglobin occupied by **8g4** was the same as that occupied by bezafibrate and adjacent to that of the natural allosteric agent 2,3-diphosphoglycerate.[2]

In the present paper we report the synthesis of a series of 2-[4-[[(arylamino)carbonyl]amino]phenoxy]-2-methylpropionic acids and their structure–allosteric activity re-

(1) Perutz, M. F.; Poyart, C. *Lancet* **1983**, 881–882.

(2) Lalezari, I.; Rahbar, S.; Lalezari, P.; Fermi, G.; Perutz, M. F. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 6117–6121.

0022-2623/89/1832-2352$01.50/0