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Specific Inhibitors of Tyrosine-Specific Protein Kinase. I. Synthesis and Inhibitory Activities of α-Cyanocinnamamides

TADAYOSHI SHIRAISHI,* KEIJI KAMEYAMA, NAOHIRO IMAI, TAKESHI DOMOTO, IKUO KATSUMI, and KIYOSHI WATANABE

Biochemical Research Laboratories, Kanegafuchi Chemical Industry Co., Ltd., Takasago, Hyogo 676, Japan

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A series of α-cyanocinnamamide derivatives was synthesized and evaluated for inhibitory activity against tyrosine-specific protein kinase using intact plasma membrane fractions from an epidermoid carcinoma cell line, A-431 cells. Among these compounds, several novel α-cyano-4-hydroxy-3,5-disubstituted cinnamamide derivatives, *e.g.*, α-cyano-3-ethoxy-4-hydroxy-5-phenylthiomethylcinnamamide (ST 638), showed potent inhibitory activity. The studies on the structure–activity relationship revealed that the presence of the hydroxy group at the 4 position and the double bond in the α-cyano-4-hydroxycinnamamide skeleton was important for potent inhibitory activity, and that the presence of hydrophobic groups at the 3 and 5 positions on the benzene ring also enhanced the inhibitory activity of α-cyano-4-hydroxycinnamamide derivatives.

Keywords——α-cyano-4-hydroxycinnamamide; α-cyano-3-ethoxy-4-hydroxy-5-phenylthiomethylcinnamamide (ST 638); tyrosine-specific protein kinase inhibition; EGF receptor kinase inhibition; structure–activity relationship

Introduction

Tyrosine-specific protein kinase (tyrosine kinase) belongs to a group of enzymes which catalyze the transfer of phosphate from adenosine triphosphate (ATP) to tyrosine residues in protein substrates. Tyrosine kinase activity has been found in several products of retroviral oncogenes, such as src,[1] yes,[2] fps,[3] abl[4], fgr[5] and erbB,[6] and in receptors for epidermal growth factor (EGF),[7] platelet-derived growth factor (PDGF),[8] insulin[9] and insulin-like growth factor.[10] Recently, tyrosine kinase activities were also found in human epidermoid tumor,[11] skin tumor[12] and brain tumor.[13] It was also reported that over-production of the EGF receptor[14] or enhanced specific activity of src molecules[15] occurs some time during the progression of normal cells to the malignant state. These findings and many other reports have suggested that tyrosine kinases play an important role in the control of cell proliferation, differentiation, carcinogenesis and progression of cancer. Therefore, tyrosine kinase inhibitors should be useful in investigating the mechanisms of carcinogenesis, and they may be effective in prevention and chemotherapy of cancer. Some tyrosine kinase inhibitors such as ATP analogs,[16] quercetin,[17] halomethylketone derivatives[18] and peptide substrate analogues[19] have been reported, but most of them seem to be weak or nonspecific inhibitors.

In the course of screening for tyrosine kinase inhibitors by using intact plasma membrane fraction of human epidermoid carcinoma cell line A-431 cells, which is a rich source of tyrosine kinase activity,[20] α-cyano-4-hydroxycinnamamide and 3,5-di-*tert*-butyl-α-cyano-4-hydroxycinnamamide, which are derivatives of the potent antiinflammatory agent α-(3,5 di-*tert*-butyl-4-hydroxybenzylidene)-γ-butyrolactone,[21] were found to possess inhibitory activity against tyrosine kinase. This prompted us to synthesize a series of α-cyano-4-hydroxy-

cinnamamide derivatives, which led to the finding of novel and potent tyrosine kinase inhibitors. This paper describes the synthesis of α-cyanocinnamamide derivatives and the determination of their inhibitory activities against tyrosine kinase.

Chemistry

4-Hydroxy-3,5-diphenylbenzaldehyde (**2**) was prepared by formylation of 2,6-diphenylphenol with hexamethylenetetramine and trifluoroacetic acid according to Smith's method.[22] 3,5-Dibenzyl-4-hydroxybenzaldehyde (**3**) was synthesized from *p*-hydroxybenzaldehyde by chloromethylation with chloromethyl methyl ether and $AlCl_3$, followed by Friedel-Crafts reaction with benzene and $SnCl_4$ (Chart 1). 3-Alkoxy-4-hydroxy-5-substituted benzaldehydes (**4**) were prepared from 3-alkoxy-4-hydroxybenzaldehydes by chloromethylation, followed by reaction with thiols, amines, and phenol (Chart 2).

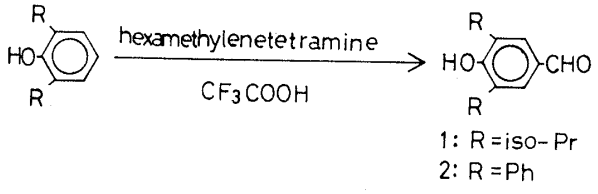


Chart 1

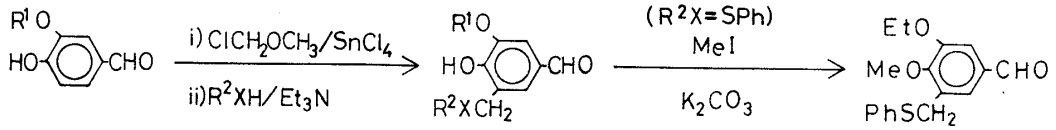


Chart 2

Most of the α-cyano-4-hydroxy-3,5-disubstituted cinnamamides (**8**—**28**) were synthesized from the aldehydes (**1**—**5**) by Knoevenagel condensation with cyanoacetamide.

4-Acyloxy derivatives (**29**, **30**) of **13** were obtained by reaction with acetic anhydride or benzoyl chloride. α-Cyanodihydrocinnamamides (**31**, **32**) were obtained by reduction of **8** and **13** with $NaBH_4$ (Chart 3).

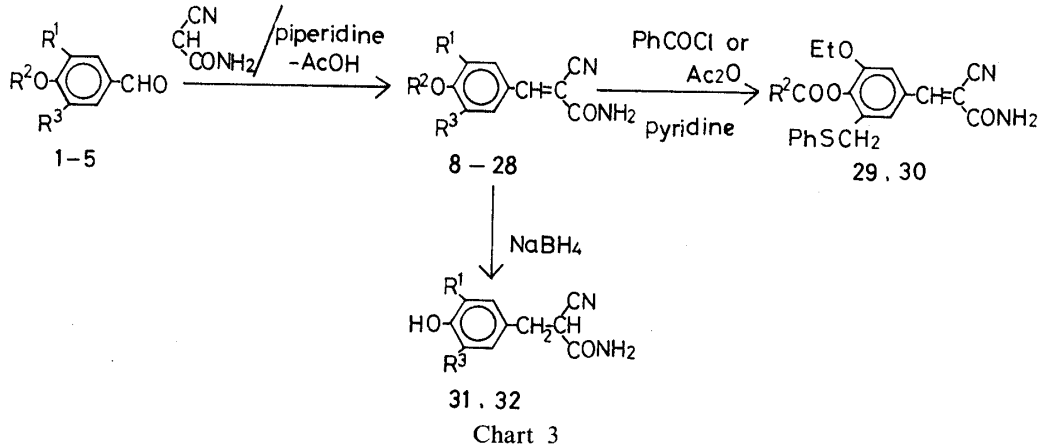
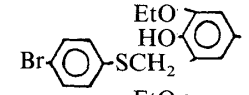
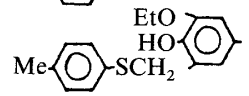
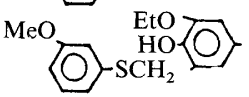
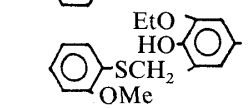
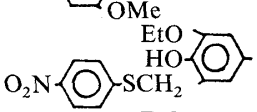
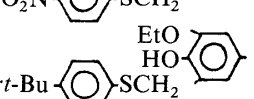
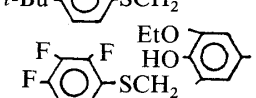
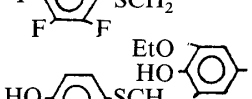
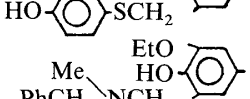
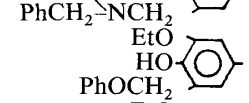
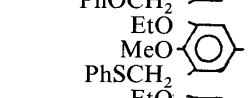
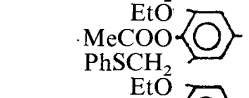
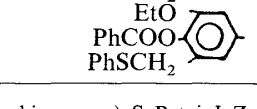


Chart 3

TABLE I. Tyrosine Kinase-Inhibitory Activity of α-Cyanocinnamamides

R-CH=C(CN)CONH$_2$

Compd. No.	R	Yield (%)	mp (°C)	Formula	Analysis (%) Calcd (Found) C	H	N	TKase Inhibition % at 0.1	1.0	10	100 (μM)	IC_{50} (μM)
6	HO		245—246 (lit. 258—259)[a]						0	58		
7	*tert*-Bu, HO, *tert*-Bu		202—203 (lit. 201—203)[b]						22	65	100	46
8	iso-Pr, HO, iso-Pr	81	170—171	$C_{15}H_{20}N_2O_2$	70.56 (70.82	7.40 7.46	10.29 10.22)	0	48	86		1.1
9	Ph, HO, Ph	32	179—181	$C_{22}H_{16}N_2O_2$	77.63 (77.66	4.74 4.70	8.23 7.95)	28	63	99		0.37
10	$PhCH_2$, HO, $PhCH_2$	73	156—158	$C_{24}H_{20}N_2O_2$	78.24 (78.10	5.47 5.54	7.60 7.46)	18	66	97		0.60
11	$PhCH_2O$, HO, $PhSCH_2$	71	164—165	$C_{24}H_{20}N_2O_3S$	69.21 (69.42	4.84 4.77	6.73 6.60)	9	55	100		0.75
12	MeO, HO, $PhSCH_2$	98	183—185	$C_{18}H_{16}N_2O_3S$	63.51 (63.28	4.74 4.75	8.23 8.34)	17	58	86		0.53
13	EtO, HO, $PhSCH_2$	84	157—161	$C_{19}H_{18}N_2O_3S$	64.39 (64.67	5.12 5.16	7.90 7.89)	31	64	100		0.37
14	EtO, HO, Cl-SCH_2	85	166—168	$C_{19}H_{17}ClN_2O_3S$	58.68 (58.80	4.40 4.29	7.20 7.24)		0	48		
15	Cl, EtO, HO, SCH_2	71	159—160	$C_{19}H_{17}ClN_2O_3S$	58.68 (58.85	4.40 4.37	7.20 7.13)		0	54		
16	Cl, Cl, EtO, HO, SCH_2	91	135—136	$C_{19}H_{16}Cl_2N_2O_3S$	53.91 (54.25	3.81 3.80	6.62 6.67)		0	69		
17	Cl, EtO, HO, SCH_2, Cl	85	192—194	$C_{19}H_{16}Cl_2N_2O_3S$	53.91 (54.13	3.81 3.90	6.62 6.24)		0	24		

18		77	171—173	$C_{19}H_{17}BrN_2O_3S$	52.66 (52.95	3.95 3.79	6.46 6.32)	0	74	
19		84	185—186	$C_{20}H_{20}N_2O_3S$	65.20 (65.50	5.47 5.37	7.60 7.49)	47	58	2.2
20		86	151—152	$C_{20}H_{20}N_2O_4S$	62.48 (62.78	5.24 5.26	7.29 6.83)	17	72	4.0
21		86	149—150	$C_{20}H_{20}N_2O_4S$	62.48 (62.26	5.24 5.07	7.29 7.11)	0	77	
22		97	182—183	$C_{19}H_{17}N_3O_5S$	57.13 (56.94	4.29 4.18	10.52 10.55)	0	42	
23		84	119—121	$C_{23}H_{26}N_2O_3S$	67.29 (67.04	6.38 6.38	6.82 6.67)	0	37	
24		98	165—166	$C_{19}H_{13}F_5N_2O_3S$	51.35 (51.65	2.95 2.97	6.30 6.02)	41	60	3.0
25		51	170—171	$C_{19}H_{18}N_2O_4S$	61.61 (61.40	4.90 4.69	7.56 7.29)	30	91	2.8
26		65	156—157	$C_{21}H_{23}N_3O_3$	69.02 (68.93	6.34 6.35	11.50 11.33)	0	52	
27		25	170—171	$C_{19}H_{18}N_2O_4$	67.45 (67.57	5.36 5.28	8.28 8.36)		18	
28		62	143—144	$C_{20}H_{20}N_2O_3S$	65.20 (65.39	5.47 5.42	7.60 7.52)		18	
29		88	178—179	$C_{21}H_{20}N_2O_4S$	63.62 (63.89	5.08 4.99	7.07 6.92)		24	
30		94	159—161	$C_{26}H_{22}N_2O_4S$	68.11 (68.29	4.84 4.86	6.11 5.80)		20	

TKase, tyrosine kinase. *a*) S. Patai, J. Zabicky, and Y. Israeli, *J. Chem. Soc.*, **1960**, 2038. *b*) I. Katumi, H. Kondo, Y. Fuse, K. Yamashita, T. Hidaka, K. Hosoe, K. Takeo, T. Yamashita, and K. Watanabe, *Chem. Pharm. Bull.*, **34**, 1610 (1986).

Results and Discussion

Tyrosine kinase-inhibitory activities of the compounds were evaluated by assaying the phosphorylation of intact plasma membrane fraction of A-431 cells; phosphorylation occurs only on tyrosine residues in the presence of EGF.

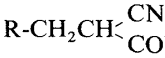
The results are summarized in Table I. The synthesized α-cyano-4-hydroxycinnamamides are pure isomers (*E* form) judging from the narrow range of melting point, the nuclear magnetic resonance (NMR) spectrum, and the fact that the Knoevenagel condensation products from aromatic aldehydes and cyanoacetamide generally have the *E* form in which the smaller cyano group is *cis* to the benzene ring.[23-25]

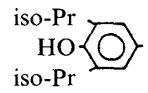
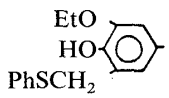
It was found that α-cyano-4-hydroxycinnamamide (**6**) and 3,5-di-*tert*-butyl-α-cyano-4-hydroxycinnamamide (**7**) have weak inhibitory activity and **7** is more active than **6**. Replacement of the *tert*-butyl groups at the 3 and 5 positions of **7** with iso-propyl (**8**), phenyl (**9**), and benzyl (**10**) and phenylthiomethyl (**11**) groups increased the inhibitory activity about 40-to120-fold in contrast to the case of compound **7**. The IC_{50} values of compounds **8**, **9**, **10** and **11** were 1.1, 0.37, 0.60 and 0.75 μM, respectively. Futhermore the replacement of the benzyloxy group at the 3 position of compound **11** with a methoxy (**12**) or ethoxy (**13**) group resulted in potent inhibitory activity with IC_{50} values of 0.53 μM for compound **12**, and 0.37 μM for compound **13**. A remarkable reduction of activity was observed upon substitution of the phenylthiomethyl group at the 5 position of compound **13** with halogen (**14**—**18**, **24**), alkyl (**19** and **23**), alkoxy (**20** and **21**), nitro (**22**), and hydroxy (**25**). However, the derivatives (**26** and **27**) in which the sulfur atom of **13** was replaced with a nitrogen atom (**26**) or oxygen atom (**27**) showed weaker inhibitory activity than **13**. Methylation (**28**) or acrylation (**29** and **30**) of **13** remarkably decreased the inhibitory activity. As shown in Table II, compounds **31** and **32** which are saturated derivatives of **7** and **13** showed only weak activity.

These results suggest that the presence of the hydroxy group at the 4 position and the double bond in the cinnamamide skeleton contributed significantly to the inhibitory activity, and the presence of hydrophobic groups at the 3 and 5 positions on the benzene ring of α-cyano-4-hydroxycinnamamide derivatives is also important for inhibitory activity.

Among the compounds tested, the most active were **9**—**12** and **13**. The inhibitory potencies of these compounds were almost equal to that of quercetin, which has been reported to be a potent inhibitor of tyrosine kinase activity associated with pp60*v-src*[17] under the same assay conditions. Since a *p*-hydroxycinnamoyl moiety is a common structure in these compounds and quercetin, this moiety may be important for potent inhibitory activity. None of these compounds showed any inhibitory effect on serine- and/or threonine-specific protein

TABLE II. Tyrosine Kinase-Inhibitory Activity of α-Cyanodihydrocinnamamides



Compd. No.	R	Yield (%)	mp (°C)	Formula	Analysis (%) Calcd (Found) C	H	N	TKase Inhibition % at 0.1	1.0	10	100 (μM)	IC_{50} (μM)
31		82	154—155	$C_{16}H_{22}N_2O_2$	70.04 (70.00)	8.08 (8.16)	10.21 (10.03)			10	51	9.8
32		87	120—121	$C_{19}H_{20}N_2O_3S \cdot 5/4\,H_2O$	60.22 (59.90)	5.98 (5.55)	7.39 (7.41)			0	29	

kinase such as adenosine 3′,5′-cyclic monophosphate (cAMP)-dependent protein kinase and Ca^{2+}/phospholipid-dependent protein kinase (data not shown). These results suggest that the compounds are potent and specific inhibitors of tyrosine kinase. In conclusion, we have found several new compounds which have potent and specific inhibitory activity against tyrosine kinase.

Experimental

All melting points are uncorrected. Infrared (IR) spectra were recorded on a Hitachi 260-30IR spectrometer. NMR spectra were recorded on a Varian EM-390NMR spectrometer with tetramethylsilane as an internal standard. Elemental analysis was performed at Sagami Chemical Research Center.

Preparation of 4-Hydroxy-3,5-diphenylbenzaldehyde (2)——A mixture of 2,6-diphenylphenol (12.3 g), hexamethylenetetramine (14 g), and trifluoroacetic acid (50 ml) was stirred at 90 °C overnight. After removal of the solvent, 3 N HCl (100 ml) was added to the residue and the mixture was stirred at 80 °C for 3 h. The resulting crystals were collected by filtration, washed with water, and dried to give **2** (9.85 g, 71.9%). IR (KBr): 3250 (OH), 1665 (CO) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 6.03 (1H, br, OH), 7.0—7.8 (12H, m, Ar-H), 9.81 (1H, s, CHO). This product was used for the next step without further purification.

Preparation of 3,5-Dibenzyl-4-hydroxybenzaldehyde (3)——$AlCl_3$ (58.7 g) was added to a solution of 4-hydroxybenzaldehyde (24.4 g) in chloromethyl methylether (200 ml) under ice cooling and the mixture was stirred at room temperature overnight. The reaction mixture was poured into ice-water (3 l) and extracted with $CHCl_3$. The organic layer was washed with water, dried with anhydrous Na_2SO_4, and concentrated under reduced pressure. The residue was dissolved in benzene (500 ml), $SnCl_4$ (52 ml) was added under stirring, and the whole was stirred at 80 °C for 4 h. The mixture was poured into ice-water (3 l) and extracted with $CHCl_3$. The organic layer was washed with water, dried with anhydrous Na_2SO_4, and evaporated under reduced pressure. The residue was purified by column chromatography (silica gel; eluent, $CHCl_3$) and recrystallized from ethyl acetate–hexane to give **3** (20.5 g, 33.9%). IR (KBr): 3170 (OH), 1655 (CO) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 4.02 (4H, s, CH_2), 5.43 (1H, br, OH), 7.1—7.6 (12H, m, Ar-H), 9.82 (1H, s, CHO). This product was used for the next step without further purification.

Preparation of 3-Alkoxy-4-hydroxy-5-substituted Benzaldehydes (4)——A typical example of the experimental procedure, used to obtain 4-hydroxy-3-methoxy-5-phenylthiomethylbenzaldehyde, is as follows. $SnCl_4$ (18 ml) was added dropwise to a solution of vanillin (7.6 g) in chloromethyl methyl ether (50 ml) under ice cooling and stirred at room temperature for 2 h. The reaction mixture was poured into ice-water (1 l). The resulting precipitate was collected by filtration, washed with water, and dried over P_2O_5 to give the corresponding 5-chlorometyl derivative (9.43 g, 94.1%). Thiophenol (2.1 ml) and Et_3N (2.8 ml) were added to a solution of the 5-chloromethyl derivative (4 g) in $CHCl_3$ (100 ml) and the mixture was stirred ar room temperature for 2 h. The reaction solution was washed with water and dried with anhydrous Na_2SO_4. After removal of the $CHCl_3$, the residue was purified by column chromatography (silica gel; eluent, $CHCl_3$–MeOH (98 : 2)) and recrystrallized from EtOH to give 3-methoxy-4-hydroxy-5-phenylthiomethylbenzaldehyde (3.28 g, 60.0%). IR (KBr): 3180 (OH), 1665 (CO) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.91 (3H, s, OCH_3), 4.18 (2H, s, SCH_2), 6.38 (1H, br, OH), 7.1—7.5 (7H, m, Ar-H), 9.72 (1H, s, CHO). This product was used for the next step without further purification. Other aldehydes were prepared similarly in 38.9—97.4% yields.

Preparation of 3-Ethoxy-4-methoxy-5-phenylthiomethylbenzaldehyde (5)——Dimethyl sulfate (0.6 ml) was added dropwise to a solution of 3-ethoxy-4-hydroxy-5-phenylthiomethylbenzaldehyde (865 mg) in 0.2 N NaOH (30 ml)–EtOH (30 ml) and the mixture was stirred at room temperature overnight. The reaction mixture was poured into ice-water and extracted with $CHCl_3$. The organic layer was washed with water and dried with anhydrous Na_2SO_4. After removal of the $CHCl_3$, the residue was purified by column chromatography (silica gel; eluent, $CHCl_3$) to give **5** (650 mg, 71.7%) as an oil. ^{1}H-NMR ($CHCl_3$) δ: 1.41 (3H, t, J=7 Hz, CH_3), 3.91 (3H, s, OCH_3), 4.03 (2H, q, J=7 Hz, CH_2), 7.0—7.5 (7H, m, Ar-H), 9.74 (1H, s, CHO). This product was used for the next step without further purification.

Preparation of α-Cyano-4-hydroxy-3,5-disubstituted Cinnamamides——A typical example of the experimental procedure, used to obtain **13**, is as follows. A solution of 3-ethoxy-4-hydroxy-5-phenylthiomethylbenzaldehyde (2.88 g), cyanoacetamide (0.84 g), acetic acid (0.5 ml), and piperidine (0.5 ml) in benzene (100 ml) was refluxed for 5 h. After cooling, the resulting precipitate was collected by filtration and recrystallized from EtOH to give **13** (2.97 g, 83.9%). IR (KBr): 3460, 3100 (OH, NH), 2200 (CN), 1695 (CO) cm^{-1}. ^{1}H-NMR ($CDCl_3$+DMSO-d_6) δ: 1.45 (3H, t, J=7 Hz, CH_3), 4.09 (2H, q, J=7 Hz, CH_2), 4.17 (2H, s, SCH_2), 7.1—7.7 (7H, m, Ar-H), 7.49 (2H, br, NH_2), 7.97 (1H, s, –CH=), 9.68 (1H, br, OH). Compounds **8**—**12**, **14**—**28** were prepared similarly in 20.5—98.2% yields.

Preparation of α-Cyano-3-ethoxy-5-phenylthiomethyl-4-substituted Cinnamamides (29 and 30)——Acetic anhydride (0.2 ml) was added to a solution of **13** (710 mg) in pyridine (10 ml), and the mixture was stirred at room temperature for 2 h, then poured into ice-water. The resulting precipitate was collected by filtration, washed with water, and recrystallized from EtOH–acetone to give **29** (700 mg, 88.3%). IR (KBr): 3400, 3125 (NH), 2210 (CN),

1760 (CO) cm^{-1}. ^{1}H-NMR ($CDCl_3$+DMSO-d_6) δ: 1.37 (3H, t, J=7 Hz, CH_3), 2.28 (3H, s, $COCH_3$), 4.05 (2H, s, SCH_2), 4.07 (2H, q, J=7 Hz, CH_2), 7.2—7.4 (5H, m, Ar-H), 7.34 and 7.58 (each 1H, d, J=2 Hz, C_2 and C_3-H), 7.48 (2H, br, NH_2), 8.02 (1H, s, CH=). Compound **30** was prepared by a similar procedure using benzoyl chloride instead of acetic anhydride, in 93.8% yield.

Preparation of α-Cyano-4-hydroxy-3,5-disubstituted Dihydrocinnamamides (31 and 32)——$NaBH_4$ (0.76 g) was added in small portions to a solution of **8** (2.75 g) in isopropanol (50 ml), and the mixture was stirred at 0—5 °C for 1 h. After additional stirring at room temperature for 2 h, the mixture was poured into ice-water. The resulting precipitate was collected by filtration, washed with water, and recrystallized from benzene to give **31** (2.26 g, 82.5%). IR (KBr): 3680, 3420, 3320, 3260, 3200 (OH, NH), 2260 (CN), 1675 (CO) cm^{-1}. ^{1}H-NMR ($CDCl_3$+DMSO-d_6) δ: 1.19 (6H, d, J=7 Hz, CH_3), 2.95 (1H, dd, J=9, 14 Hz, CH_2-CH), 3.09 (1H, dd, J=6, 14 Hz, CH_2-CH), 3.29 (2H, sep, J=7 Hz, CH_3CHCH_3), 3.72 (1H, dd, J=6, 9 Hz, CH_2-CH), 6.87 (2H, s, Ar-H), 7.09 (1H, br, NH), 7.52 (1H, br, OH), 7.57 (1H, br, NH). Compound **32** was prepared by a similar procedure in 87.1% yield.

Biochemical Tests

Cell Culture and Preparation of Plasma Membrane Fraction of A-431 Cells——Human epidermoid carcinoma A-431 cells were kindly supplied by Dr. K. Onodera, University of Tokyo. The cells were grown in Dulbecco's modified Eagle's medium (Nissui) supplemented with 10% fetal calf serum (Flow Laboratories), penicillin G (50 IU/ml), streptomycin (50 μg/ml) and kanamycin (25 μg/ml) for 6 d at 37 °C in a CO_2 incubator (5% CO_2 in air). Intact plasma membrane fraction from A-431 cells was prepared by the method of Carpenter *et al.*[26] In brief, the cells were scraped from the culture flask, concentrated into a small volume by centrifugation and lysed by dilution into a hypotonic borate/ethylenediaminetetraacetic acid (EDTA) buffer, pH 10.2. The lysed cells were filtered through a nylon screen and a crude membrane fraction was obtained by centrifugation at 25000 *g* for 30 min. The pellet was resuspended, layered over 35% (w/v) sucrose, and centrifuged (40000 *g* for 45 min) in a swinging bucket rotor. The material at the buffer–sucrose interface was collected with a Pasteur pipette, suspended in 10 mM Hepes buffer, pH 7.4, and recentrifuged (75000 *g* for 30 min). The final membrane preparation was resuspended in Hepes buffer (approximately 10 mg of protein/ml), and stored at −70 °C until use. The tyrosine kinase activity of the plasma membrane fraction was confirmed by phosphorylation assay and also by phospho amino acid analysis. The prepared plasma membrane fraction was phosphorylated only at tyrosine residues, when phosphorylated in the presence of EGF, as reported by Carpenter *et al.*[26] Protein concentration was determined as reported by Bradford.[27]

Standard Phosphorylation Assay——Phosphorylation of proteins was performed as described by Carpenter *et al.*[26] The incubation system (70 μl) contained 20 mM-Hepes buffer (pH 7.4), 7.5 μg of bovine serum albumin, intact plasma membrane fraction (2—10 μg protein), 100 ng of EGF and [γ-^{32}P]ATP (5000 Ci/mmol, Amersham Japan). Either dimethylsulfoxide (DMSO) or a test compound dissolved in DMSO was added to the reaction mixture to a final concentration of 0.2%. The reaction mixture was incubated with or without EGF for 15 min at 0 °C. Then the phosphorylation reaction was initiated by adding [γ-^{32}P]ATP (0.1—0.3 μCi). After incubation at 0 °C for 15 min, 50 μl aliquots of the reaction mixture were withdrawn and spotted on Whatman 3MM filter papers (2.4 cm diameter) which were immediately dropped into a beaker containing a cold solution of 10% trichloroacetic acid (TCA)/10 mM sodium pyrophosphate. Papers were washed with three changes of cold 10% TCA/10 mM sodium pyrophosphate, then one of ethanol and dried. The radioactivity of the acid-insoluble precipitate on papers was determined in a liquid scintillation counter. The percent inhibition of each compound was calculated by means of the following equation.

$$\text{inhibition } \% = 1-(a-b)/(c-d)\times 100$$

a, radioactivity in the reaction mixture containing test compound with EGF; b, radioactivity in the reaction mixture containing test compound without EGF; c, radioactivity in the control reaction mixture with EGF; d, radioactivity in the control reaction mixture without EGF.

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