# Structural Studies on Bioactive Compounds. 23. Synthesis of Polyhydroxylated 2-Phenylbenzothiazoles and a Comparison of Their Cytotoxicities and Pharmacological Properties with Genistein and Quercetin

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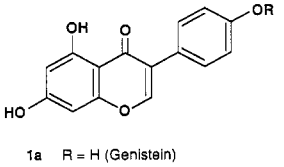
A series of polyhydroxylated 2-phenylbenzothiazoles **3** has been prepared by demethylation of the precursor methoxylated 2-phenylbenzothiazoles **9**. The key step in the construction of the benzothiazole nucleus involves a Jacobson cyclization of methoxylated thiobenzanilides **8**. The target compounds inhibit WiDr human colon tumor cells and MCF-7 human mammary tumor cells *in vitro* with $IC_{50}$ values in the low micromolar range, but the activity against MCF-7 cells is not related to estrogen receptor-binding affinity. None of the compounds showed selective cytotoxicity against Abelson virus-transformed ANN-1 cells encoded with the pp120$^{gag\text{-}abl}$ tyrosine kinase compared with the parental 3T3 line. Compounds were only marginally inhibitory to the EGF receptor-associated protein tyrosine kinase from a membrane preparation of A431 cells. The most active compound was 4,6-dihydroxy-2-(4-hydroxyphenyl)benzothiazole (**3b**) which has the same overall hydroxyl substitution pattern as genistein (**1a**). The compounds were weakly cytotoxic for an EGF receptor, overexpressing cell line HN5, but when tested for differential toxicity against the EGF receptor tyrosine kinase or the PDGF receptor tyrosine kinase in a standard mitogenesis assay utilizing human fibroblasts, no discrimination was observed. In this assay, the compounds inhibited DNA synthesis when added to cells during S phase. This suggests that inhibition could not be interpreted in terms of tyrosine kinase inactivation but more likely as a relatively broad specificity for the ATP-binding domain of other kinases such as thymidine kinase.

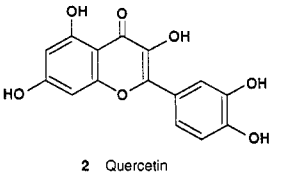
## Introduction

The isoflavone genistein (**1a**)[1] and the flavone quercetin (**2**)[2] are competitive inhibitors at the ATP-binding site of kinases, and synthetic flavones modeled on quercetin have been investigated recently in an effort to identify compounds with greater inhibitory selectivity toward tyrosine kinases over serine–threonine kinases.[3,4]




1a R = H (Genistein)

1b R = Me (Biochanin A)




2 Quercetin

Protein tyrosine kinases occupy a central position in the control of cellular proliferation. Several transforming oncogenes (e.g., *src*, *abl*) are known to possess tyrosine kinase activity, and it is well recognized that the response of many cells to growth factors is initiated by activation of receptor tyrosine kinases (RTKs). Overexpression of certain RTKs shows association with promotion and maintenance of malignant disease. For example, the epidermal growth factor (EGF) receptor is frequently expressed at high levels in certain carcinomas and shows an inverse correlation with survival (particularly breast and bladder cancers).[5] Thus, inactivation of the specific tyrosine kinases that are responsible for the malignant phenotype of certain cancers represents a potential approach for design of antiproliferative drugs.[6]

We have recently solved the crystal structure of 5,6-dimethoxy-2-(4-methoxyphenyl)benzothiazole (**9i**)[7] and used the structural information to model comparisons between polyhydroxylated 2-phenylbenzothiazoles and the adenine fragment of ATP. These preliminary investigations suggest that suitably substituted benzothiazoles might mimic the ATP-competitive binding of genistein and quercetin to tyrosine kinases. However, we are aware that genistein and quercetin have disparate effects on cells and linking the two natural products as though they were a single lead compound for drug design purposes may not be entirely appropriate; for example, the $G_2$-M arrest initiated by genistein on human gastric HGC-27 cells is an effect unique among structurally related flavanoids.[8]

In addition to their potential tyrosine kinase-inhibitory activity, hydroxylated 2-phenylbenzothiazoles of general structure **3** are structural analogues of the hydroxylated 1,3-dialkyl-2-phenylindoles **4**,[9] 3-alkyl-2-phenylbenzo[*b*]-thiophenes **5**,[10] and 3-alkyl-2-phenylbenzo[*b*]furans **6**[11] which have been studied extensively by von Angerer and his colleagues. In the latter heterocycles, *in vitro* cytotoxicity strongly correlates with estrogen receptor-binding affinity, and the target compounds **3** might be anticipated to exhibit similar effects. However, a recent study comparing the activity of genistein on human breast

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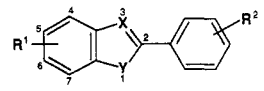


3 X = N, Y = S

4 X = C–Alk, Y = N–Alk

5 X = C–Alk, Y = S

6 X = C–Alk, Y = O

$R^1$ and $R^2$ = H, OH or $(OH)_2$

carcinoma cell lines MCF-7 ($E^+$) and MDA-468 ($E^-$) has confirmed that the presence of estrogen receptors is not a requirement for activity of the isoflavone.[12]

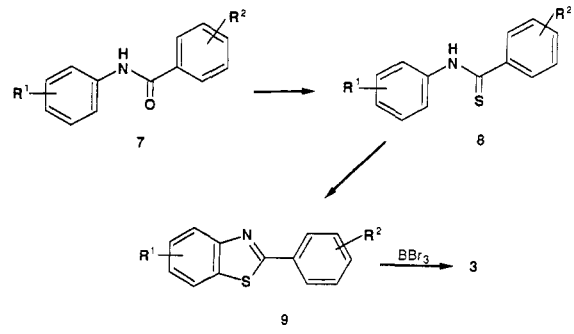
Several polyhydroxylated 2-phenylbenzothiazoles, some with hydroxyl substitution patterns similar to those in genistein and quercetin, have been synthesized and subjected to a broad *in vitro* evaluation. Compounds were tested for cytotoxicity against virally transformed cells and cancer or normal cell lines. These included murine fibroblasts transformed by Abelson murine leukemia virus which express the tyrosine kinase pp120$^{gag\text{-}abl}$, human MCF-7 breast carcinoma cells which express the EGF receptor (EGFR) as well as the estrogen receptor, human WiDr colon cells, human HN5 squamous carcinoma cells which overexpress the EGFR, and a normal human fibroblast cell line which expresses both the EGFR and PDGF receptor. Certain compounds were also evaluated for their ability to inhibit EGF-stimulated EGFR tyrosine kinase activity in a membrane preparation from A431 cells. When supplies allowed, compounds were also tested for estrogen receptor-binding affinity (RBA) and aromatase-inhibitory activity.

## Chemistry

The simplest route to substituted 2-phenylbenzothiazoles involves variations of the reaction between *o*-aminothiophenols and substituted benzoic acid derivatives (e.g., in polyphosphoric acid).[13] However, these routes are not readily adaptable to the preparation of the polyhydroxylated target compounds, especially where hydroxyl substitution in the benzothiazole nucleus is required. Instead, the starting materials were the known methoxy-substituted benzanilides **7** which were readily available from Schotten–Baumann reactions; these benzanilides were converted to thiobenzanilides **8** by Lawesson's reagent. Cyclization of the thiobenzanilides to benzothiazoles **9** was accomplished by potassium ferricyanide in aqueous sodium hydroxide (Jacobson synthesis),[14] and finally, demethylation of the methyl ethers was effected by boron tribromide in dichloromethane at −70 °C to afford the target hydroxylated benzothiazoles **3** (Scheme 1).

The thionation of benzanilides with Lawesson's reagent was accomplished in refluxing chlorobenzene. In the majority of cases, products crystallized out on cooling; to recover the more soluble thiobenzanilides, excess chlorobenzene was removed by steam distillation. Yields and physical characteristics of the products are recorded in Table 1. Jacobson synthesis of methoxy-substituted benzothiazoles **9** proceeded generally in high yields (70–90%), and the scope of the synthesis was broad (Table 2). Cyclization of 3,4′-dimethoxythiobenzanilide (**8h**) with a substituent *meta* to the anilide N atom afforded an

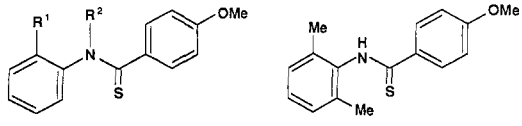
**Scheme 1**[a]



[a] $R^1$ and $R^2$ = H, OMe, or $(OMe)_2$.

approximately 1:1 mixture of the 5-methoxybenzothiazole **9g** and its 7-methoxy isomer (**9p**). However, thiobenzanilides **8i,j,k** with a 3,4-disubstitution pattern in the anilide moiety gave only 5,6-dimethoxybenzothiazoles **9h,i,j**, respectively, as isolated products.

The mechanism of the cyclization of thiobenzanilides **8** to 2-phenylbenzothiazoles **9** probably involves a one-electron oxidation of the thiolate anions **10** to give the thiol radicals **11** which then attack the unoccupied *ortho* position in the substrates. Elimination of a hydrogen radical from the reactive intermediates (**12**) effects aromatization to the benzothiazoles **9** (Scheme 2). The significance of the availability of a free NH group to facilitate thione–thiolate conversion in thiobenzanilides was emphasized by our failure to cyclize the *N*-alkylthiobenzanilide **13a**, the dihydroindole **13b**, and the tetrahydroquinoline **13c** with alkaline potassium ferricyanide: these modified substrates have a fixed thione configuration. 2,6-Dimethylthiobenzanilide **14**, in which the *ortho* positions in the thiobenzanilide are blocked, also failed to cyclize.



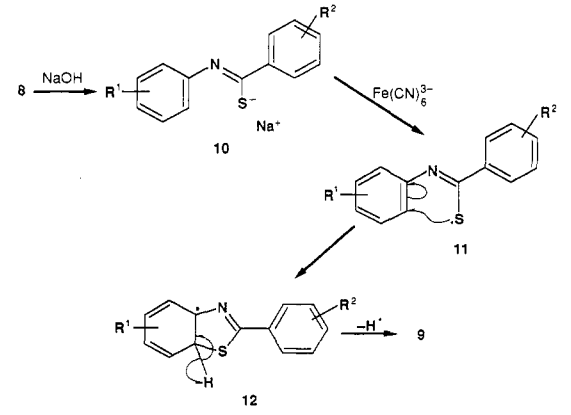
13a $R^1$ = H, $R^2$ = Me

13b $R^1$ $R^2$ = $(CH_2)_2$

13c $R^1$ $R^2$ = $(CH_2)_3$

14

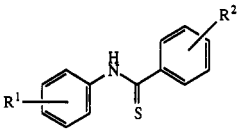
**Scheme 2**[a]



[a] $R^1$ and $R^2$ = H, OMe, or $(OMe)_2$.

Demethylation of the methoxybenzothiazoles **9** proceeded cleanly in most cases with boron tribromide in dichloromethane at −70 °C to give moderate yields of hydroxybenzothiazoles (Table 3), but compounds with four

**Table 1.** Structure, Yields, and Physical Characteristics of Thiobenzanilides **8**

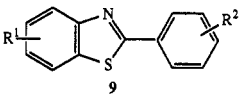


**8**

| compd | $R^1$ | $R^2$ | yield (%) | mp (°C) | formula | analysis[a,b] |
|---|---|---|---|---|---|---|
| **8a** | H | 3-OMe | 74 | 95–96 | $C_{14}H_{13}NOS$ | |
| **8b** | 2-OMe | H | 76 | 121–122 | $C_{14}H_{13}NOS$ | |
| **8c** | 2-OMe | 4-OMe | 25 | 69–71 | $C_{15}H_{15}NO_2S$ | C,H,N |
| **8d** | 2-Me | 4-OMe | 65 | 119–120 | $C_{15}H_{15}NOS$ | |
| **8e** | 2,4-di-OMe | 4-OMe | 43 | 103–104 | $C_{16}H_{17}NO_3S$ | C,H,N |
| **8f** | 2,4-di-OMe | 3,4-di-OMe | 84 | 128–129 | $C_{17}H_{19}NO_4S$ | C,H,N |
| **8g** | 3-OMe | H | 62 | 81–82 | $C_{14}H_{13}NOS$ | C,H,N |
| **8h** | 3-OMe | 4-OMe | 24 | 98–99 | $C_{15}H_{15}NO_2S$ | C,H,N |
| **8i** | 3,4-di-OMe | H | 75 | 154–155 | $C_{15}H_{15}NO_2S$ | C,H,N |
| **8j** | 3,4-di-OMe | 4-OMe | 83 | 149–151 | $C_{16}H_{17}NO_3S$ | C,H,N |
| **8k** | 3,4-di-OMe | 3,4-di-OMe | 86 | 165–166 | $C_{17}H_{19}NO_4S$ | C,H,N |
| **8l** | 3,5-di-Ome | H | 74 | 135–136 | $C_{15}H_{15}NO_2S$ | C,H,N |
| **8m** | 3,5-di-OMe | 3,4-di-OMe | 75 | 155–156 | $C_{17}H_{19}NO_4S$ | C,H,N |
| **8n** | 4-OMe | H | 79 | 131–133 | $C_{14}H_{13}NOS$ | C,H,N |
| **8o** | 4-OMe | 3-OMe | 47 | 75–76 | $C_{15}H_{15}NO_2S$ | C,H,N |
| **8p** | 4-OMe | 4-OMe | 89 | 151–152 | $C_{15}H_{15}NO_2S$ | C,H,N |
| **8q** | 4-OMe | 3,4-di-OMe | 87 | 154–155 | $C_{16}H_{17}NO_3S$ | C,H,N |
| **8r** | 4-Cl | 4-OMe | 91 | 181–183 | $C_{14}H_{12}ClNOS$ | C,H,N |

[a] Analyses of all new compounds agree within ±0.4% of calculated values for indicated atoms. [b] Compounds were also characterized fully by UV, IR, and $^1H$ NMR spectra.

**Table 2.** Structure, Yields, and Physical Characteristics of Methoxy-Substituted Benzothiazoles **9**



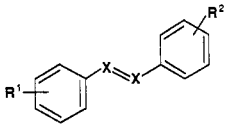
**9**

| compd | $R^1$ | $R^2$ | yield (%) | mp (°C) | formula | analysis[b,c] |
|---|---|---|---|---|---|---|
| **9a** | H | 3-OMe | 80 | 81–22 | $C_{14}H_{11}NOS$ | |
| **9b** | H | 4-OMe | 90[a] | 121–122 | $C_{14}H_{11}NOS$ | |
| **9c** | 4-OMe | H | 76 | 103–104 | $C_{14}H_{11}NOS$ | C,H,N |
| **9d** | 4-Me | 4-OMe | 90 | 87–88 | $C_{15}H_{13}NOS$ | C,H,N |
| **9e** | 4,6-di-OMe | 4-OMe | 66 | 123–124 | $C_{16}H_{15}NO_3S$ | C,H,N |
| **9f** | 4,6-di-OMe | 3,4-di-OMe | 48 | 148–149 | $C_{17}H_{17}NO_4S$ | C,H,N |
| **9g** | 5-OMe | 4-OMe | 23 | 120–121 | $C_{15}H_{13}NO_2S$ | C,H,N |
| **9h** | 5,6-di-OMe | H | 40 | 142–144 | $C_{15}H_{13}NO_2S$ | |
| **9i** | 5,6-di-OMe | 4-OMe | 91 | 159–160 | $C_{16}H_{15}NO_3S$ | C,H,N |
| **9j** | 5,6-di-OMe | 3,4-di-OMe | 47 | 176–177 | $C_{17}H_{17}NO_4S$ | C,H,N |
| **9k** | 6-OMe | H | 73 | 114–115 | $C_{14}H_{11}NOS$ | C,H,N |
| **9l** | 6-OMe | 3-OMe | 71 | 98–99 | $C_{15}H_{13}NO_2S$ | C,H,N |
| **9m** | 6-OMe | 4-OMe | 71 | 156–158 | $C_{15}H_{13}NO_2S$ | |
| **9n** | 6-OMe | 3,4-di-OMe | 86 | 123–124 | $C_{16}H_{15}NO_3S$ | C,H,N |
| **9o** | 6-Cl | 4-OMe | 90 | 145–146 | $C_{14}H_{10}ClNOS$ | C,H,N |
| **9p** | 7-OMe | 4-OMe | 29 | 140–141 | $C_{15}H_{13}NO_2S$ | C,H,N |

[a] Prepared from 2-aminothiophenol and 4-methoxybenzoyl chloride in pyridine (see the Experimental Section). [b] Analyses of all new compounds agree within ±0.4% of calculated values for indicated atoms. [c] Compounds were also characterized fully by UV, IR, and $^1H$ NMR spectra.

methoxy substituents (**9f,j**) required the use of a large excess of boron tribromide. Unfortunately, only one example of a 5,6-dihydroxybenzothiazole (**3e**) was available in sufficient quantity for biological evaluation. Genistein (**1a**) was also formed (56%) by demethylation of its methyl ether Biochanin A (**1b**).

To complete the synthetic work, the trihydroxyazobenzene **15a** was prepared by coupling diazotized 4-aminophenol with resorcinol. This azo compound in the *E*-configuration is structurally related to the *E*-stilbenes diethylstilbestrol (**15b**) and piceatannol (**15c**)and also to the 2-arylbenzothiazoles where the sulfur atom can be envisaged as fixing the azomethine linkage in the *E*-configuration. Piceatannol, an antileukemic principle from seeds of *Euphorbia lagascae*, inhibits $p56^{lck}$ protein tyrosine kinase by competing at the protein substrate-binding locus rather than the ATP-binding site,[15] and analogues of piceatannol have been synthesized with enhanced potency over the lead natural product.[16]



**15a** X = N; $R^1$ = 2,4-di-OH; $R^2$ = 4-OH

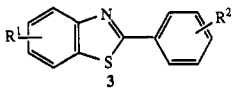
**15b** X = CEt; $R^1$ = $R^2$ = 4-OH

**15c** X = CH; $R^1$ = 3,5-di-OH; $R^2$ = 3,4-di-OH

## Biological Results and Discussion

Results of the *in vitro* cytotoxicity tests against murine and human cell lines are presented in Table 4. No compound showed significantly greater cytotoxicity toward

**Table 3.** Structure, Yields, and Physical Characteristics of Hydroxy-Substituted Benzothiazoles **3**



**3**

| compd | $R^1$ | $R^2$ | yield (%) | mp (°C) | formula | analysis[c,d] |
|---|---|---|---|---|---|---|
| **3a** | H | 4-OH | 56 | 220–221 | $C_{13}H_9NOS$ | |
| **3b** | 4,6-di-OH | 4-OH | 51 | 236–237 | $C_{13}H_9NO_3S$ | C,H,N |
| **3c**[a] | 4,6-di-OH | 3,4-di-OH | 48 | >330[b] | $C_{13}H_9NO_4S$ | C,H,N |
| **3d** | 5-OH | 4-OH | 80 | 234–235 | $C_{13}H_9NO_2S$ | C,H,N |
| **3e** | 5,6-di-OH | 4-OH | 36 | >335[b] | $C_{13}H_9NO_3S$ | C,H,N |
| **3f**[a] | 5,6-di-OH | 3,4-di-OH | 94 | >320[b] | $C_{13}H_9NO_4S$ | C,H,N |
| **3g** | 6-OH | H | 50 | 223–224 | $C_{13}H_9NOS$ | C,H,N |
| **3h** | 6-OH | 3-OH | 73 | 200–201 | $C_{13}H_9NO_2S$ | C,H,N |
| **3i** | 6-OH | 4-OH | 65 | 275–276 | $C_{13}H_9NO_2S$ | C,H,N |
| **3j** | 6-OH | 3,4-di-OH | 21 | 261–265[b] | $C_{13}H_9NO_3S$ | C,H,N |

[a] 20 mol equiv of boron tribromide used to effect demethylation. [b] Melts with decomposition. [c] Analyses of all new compounds agree within ±0.4% of calculated values for indicated atoms. [d] Compounds were also characterized fully by UV, IR, and $^1H$ NMR spectra.

the tyrosine kinase-encoded murine ANN-1 cell line over the 3T3 line. Those benzothiazoles with only one hydroxyl group in the phenyl (**3a**) or benzothiazole (**3g**) nucleus were the least cytotoxic, and compounds **3e,i,j** were the most cytotoxic with $IC_{50}$ values in the range 5.5–13.5 μM. 4,6-Dihydroxy-2-(4-hydroxyphenyl)benzothiazole (**3b**) and genistein (**1a**) which have a similar substitution pattern of hydroxyl groups were approximately equipotent against both cell lines. The lead compound quercetin (**2**) has been shown previously to exhibit no differential cytotoxicity to ANN-1 cells.[4] The most potent compounds tested against the murine cell lines were the azo compound **15a** and the reference compound diethylstilbestrol (**15b**). Compound **15a** also has the same arrangement of hydroxyl groups as genistein but is 4-fold more cytotoxic than the isoflavone toward the ANN-1 cells. Tamoxifen also showed activity ($IC_{50}$ = 4 μM) against both cell lines.

There were no striking divergences in cytotoxicity between compounds against the WiDr human colon cell line with a mean $IC_{50}$ value of 39 μM (Table 4). The MCF-7 human mammary cell line was, in general, more sensitive to all the compounds (mean $IC_{50}$ = 19 μM) with the most potent derivative being 6-hydroxy-2-(3-hydroxyphenyl)-benzothiazole (**3h**) ($IC_{50}$ = 2.7 μM) which was approximately 10-fold more cytotoxic than the 6-hydroxy-2-(4-hydroxyphenyl)benzothiazole isomer (**3i**). However, this difference cannot be correlated directly with relative binding affinity (RBA) for the estrogen receptor obtained from calf uterus (RBA for estradiol = 100) since compound **3h** has weaker affinity (RBA = 0.24) than **3i** (RBA = 0.70). Introduction of additional hydroxyl groups decreased or abolished estrogen receptor binding. As expected, compounds **3a,g** with only one hydroxyl group showed no binding affinity (RBA = <0.01), whereas the compound with the highest RBA prepared in the present work was the trihydroxyazobenzene **15a** (RBA = 0.78).

Direct comparisons can be made between binding affinities for the estrogen receptor of hydroxylated 2-phenylbenzothiazoles and the 3-alkyl-2-phenylbenzo[*b*]-thiophens **5** recently reported by von Angerer.[10] The benzo[*b*]thiophens as a group clearly outrank all the benzothiazoles in receptor-binding affinity. Notably, 3-ethyl-5-hydroxy-2-(4-hydroxyphenyl)benzo[*b*]thiophen (**5**; $R^1$ = 5-OH, $R^2$ = 4-OH, X = C-Et, Y = S) (RBA = 60) and the 6-hydroxy-2-(4-hydroxyphenyl)benzo[*b*]-thiophen isomer (RBA = 28) are particularly potent. This may be attributable to the presence of a hydrophobic alkyl group on the central thiophen residue which may be important for potent estrogen receptor binding.[10]

**Table 4.** Cytotoxicities of Hydroxy-Substituted Benzothiazoles and Related Compounds in Mouse and Human Cell Lines

| | $IC_{50}$ (μM)[a] | | | |
|---|---|---|---|---|
| compd | 3T3 | ANN-1 | MCF-7 | WiDr |
| **1a** (genistein) | 24.0 | 8.0 | 15.1 | 27.7 |
| **2** (quercetin) | 39.5 | 32.5 | 24.0 | 40.2 |
| **3a** | 71.5 | 77.0 | 27.0 | 68.7 |
| **3b** | 20.0 | 21.5 | 33.3 | 31.2 |
| **3c** | 47.0 | 58.5 | 41.3 | 47.7 |
| **3d** | 25.0 | 25.5 | 41.4 | 62.2 |
| **3e** | 13.5 | 9.0 | 8.4 | 24.7 |
| **3f** | 56.5 | 32.0 | 5.1 | 34.2 |
| **3g** | 79.5 | 71.0 | 27.3 | 45.2 |
| **3h** | 30.5 | 17.0 | 2.7 | 18.3 |
| **3i** | 11.0 | 13.5 | 25.0 | 67.2 |
| **3j** | 6.0 | 5.5 | 17.6 | 23.0 |
| **15a** | 5.5 | 2.0 | 7.0 | 27.0 |
| **15b** (diethylstilbestrol) | 2.5 | 3.0 | 5.0 | ND |

[a] Mean of two determinations (3T3 and ANN-1) or single determination (MCF-7 and WiDr). All tests were performed at least in duplicate. ND = not determined.

Quercetin has been reported to inhibit human placental aromatase with an $IC_{50}$ of 12 μM.[17] Compounds **3c,g,i** and genistein were assayed against aromatase enzyme in human placental microsomes using [1-$^3H$]androsterone as substrate. Compounds were tested at 2, 5, and 10 μM, but no inhibition was observed at these concentrations (results not shown).

Several of the polyhydroxylated 2-phenylbenzothiazoles were compared with genistein and quercetin for their ability to inhibit EGF-stimulated protein tyrosine kinase activity of EGFR derived from a membrane preparation of A431 cells (Table 5). All compounds were tested at a concentration of 150 μM, and overall, the compounds were inactive or of low potency: the dihydroxy compounds **3d,h** had no inhibitory properties at this concentration, whereas 6-hydroxy-2-(4-hydroxyphenyl)benzothiazole (**3i**), the trihydroxybenzothiazole **3b**, and the tetrahydroxy compounds **3c,f** were approximately equipotent with quercetin but less active than genistein when evaluated in the same test. Because of the disappointing activities (marginal at best) of polyhydroxylated benzothiazoles in this isolated EGFR preparation, we were unable to correlate activities with cytotoxic effects in cellular systems.

Squamous carcinoma cells such as HN5 cells overexpress the EGFR and are dependent on autocrine stimulation of this receptor for growth.[18] When selected polyhydroxylated 2-phenylbenzothiazoles were tested for their ability to block growth of HN5 cells, they were found to be

**Table 5.** Inhibition of the Protein Tyrosine Kinase Activity of EGF Receptor Derived from A431 Cells by Hydroxy-Substituted Benzothiazoles, Genistein, and Quercetin

| compd | concentration of compound (μM) | inhibition (%) |
|---|---|---|
| **1a** (genistein) | 150 | 63[a] |
| **2** (quercetin) | 150 | 30 |
| | 50 | 25[b] |
| **3b** | 150 | 47 |
| **3c** | 150 | 36 |
| **3d** | 150 | 5 |
| **3f** | 150 | 38 |
| **3h** | 150 | -4 |
| **3i** | 150 | 42 |

[a] The discrepancy between the activity of genistein implied in this test and a published $IC_{50}$ value of 0.7 (μM)[23] is probably due to differences in the methods used to prepare membranes from A431 cells. [b] Data from ref 4.

**Table 6.** Effect of Hydroxy-Substituted Benzothiazoles on (i) Growth of HN5 Squamous Carcinoma Cells and (ii) Ability of Growth Factors To Initiate DNA Synthesis in Density-Arrested Human Foreskin Fibroblasts

(i) HN5 Squamous Carcinoma Cells

| compd | $IC_{50}$ (μM) |
|---|---|
| **2** (quercetin) | 190 |
| **3b** | 300 |
| **3c** | 130 |
| **3i** | 130 |
| **15a** | 58 |

(ii) Density-Arrested Human Foreskin Fibroblasts

| | $IC_{50}$ (μM) | | |
|---|---|---|---|
| compd | EGF[a] | EGF[b] | PDGF[c] |
| **2** (quercetin) | 46 | 28 | 100 |
| **3b** | 16 | 7 | 20 |
| **3c** | 9 | 7 | 68 |
| **3i** | 6.5 | 13 | 14 |
| **15a** | 37 | 9 | 75 |

[a] Test compounds were added to cells at the same time as 0.75 ng/mL EGF. [b] Test compounds were added to cells 22 h after addition of 0.75 ng/mL EGF. [c] Test compounds were added at the same time as 2.5% human serum used as a source of PDGF.

relatively ineffective; the most potent agent was the azo compound **15a** with an $IC_{50}$ of 58 μM (Table 6i).

A comparison of the sensitivities of the EGF and PDGF receptor tyrosine kinases to the test compounds was performed using density-arrested human foreskin fibroblasts in a standard mitogenesis assay. In the first series of experiments, cells were treated with the drug substance simultaneously with EGF at a concentration of the growth factor (0.75 ng $mL^{-1}$) which gives 50% maximum stimulation or with human serum (2.5%) acting as a source of PDGF. Growth was assessed by $^{125}$IUdR uptake after 24 h. In all cases, the compounds antagonized the EGF-stimulated growth ($IC_{50}$ = 6.5–46 μM) slightly more than the human serum-stimulated growth ($IC_{50}$ = 14–100 μM). In these experiments, the most active derivatives were **3c** ($IC_{50}$ = 9 μM) and **3i** ($IC_{50}$ = 6.5 μM); the reference flavone quercetin (**2**) was the least active of the compounds studied (Table 6ii). In the second series of experiments, the fibroblasts were stimulated with EGF and the test compounds administered 22 h later when the cells were in S phase (i.e., the growth factor was allowed to initiate DNA synthesis before application of the test compound). In these experiments, the compounds were found to be as potent as when added simultaneously with the growth factor, indicating that they were acting at one (or more) site downstream of the receptor tyrosine kinase. As the compounds were designed to mimic the ATP-binding site of kinases and as the assay depends on uptake and phosphorylation of thymidine, the latter being catalyzed by thymidine kinase, it is possible that the inhibitory effect of the compounds can be attributed to an effect on this enzyme. Thymidine kinase occurs in two forms in human tissue;[19] the susceptibility of these isoforms to inhibition by polyhydroxylated 2-arylbenzothiazoles, genistein, and quercetin is an area to be investigated. Also, this work has shown that the azo compound **15a** has a rather broad spectrum of pharmacological properties which are worthy of detailed evaluation.

In conclusion, these results suggest that the cytotoxicities exhibited by this new series of compounds in a range of cell types cannot be ascribed to inhibition of a specific tyrosine kinase. Moreover, in view of the potential effects of genistein, quercetin, and these new compounds on other cellular processes and DNA metabolism, caution must be taken in interpreting experimental data for similar compounds.

## Experimental Section

All new compounds listed in the tables were characterized by elemental microanalysis (C, H, and N values ±0.4% of theoretical values) and mass spectrometry (recorded on a V.G. Micromass 12 instrument at 70 eV; source temperature 250–300 °C). UV spectra were recorded on a Pye Unicam SP8000 spectrometer in 95% ethanol solutions. IR spectra were determined on a Perkin-Elmer 1310 infrared spectrometer as either Nujol mulls or KBr discs. $^1$H NMR spectra were recorded in $CDCl_3$ (unless otherwise indicated) on a Bruker AM300 spectrometer. TLC systems for routine monitoring of reaction mixtures and confirming the homogeneity of analytical samples employed Kieselgel $60F_{254}$ (0.25 mm) with either $CHCl_3$ or $CHCl_3$–2% ethanol as developing solvents. Sorbsil silica gel C 60-H (40–60 μm) was used for flash chromatographic separations.

**General Method for the Synthesis of Methoxy-Substituted Thiobenzanilides 8.** Appropriately substituted benzanilides **7** (0.01 M) and Lawesson's reagent (0.6 mol equiv) were boiled (3 h) in sufficient chlorobenzene (5–10 mL) to produce a clear solution. The solution was cooled; products were collected and crystallized from methanol or ethanol. Yields and physical characteristics are recorded in Table 1.

***N*-(4-Methoxythiobenzoyl)methylaniline (13a).** Reaction of *N*-(4-methoxybenzoyl)methylaniline with Lawesson's reagent according to the general method (above) afforded the thiobenzoylmethylaniline (50%) as a yellow solid, mp 100–101 °C (Found: C, 69.8; H, 5.8; N, 5.5; $M^+$, 257. $C_{15}H_{15}NOS$ requires C, 70.0; H, 5.8; N, 5.4; M, 257). $^1$H NMR δ 3.85 (3H, s, OMe), 3.65 (3H, s, NMe).

**2,3-Dihydro-1-(4-methoxythiobenzoyl)indole (13b).** Similarly prepared, from 2,3-dihydro-1-(4-methoxybenzoyl)indole and Lawesson's reagent, the thiobenzoylindole (42%) crystallized from methanol as yellow needles, mp 120–122 °C (Found: $M^+$, 239. $C_{15}H_{13}NOS$ requires M, 237). $^1$H NMR (DMSO-$d_6$) δ 3.80 (3H, s, OMe). $\lambda_{max}$ 230, 292, 326 nm.

**1,2,3,4-Tetrahydro-1-(4-methoxythiobenzoyl)quinoline (13c).** Prepared from 1,2,3,4-tetrahydro-1-(4-methoxybenzoyl)-quinoline and Lawesson's reagent, the quinoline (58%) crystallized from methanol as yellow prisms, mp 146–147 °C (Found: C, 71.8; H, 5.9; N, 5.0. $C_{17}H_{17}NOS$ requires C, 72.1; H, 6.0; N, 4.9). $^1$H NMR (DMSO-$d_6$) δ 3.70 (3H, s, OMe). $\lambda_{max}$ 210, 310 nm.

**2,6-Dimethyl-*N*-(4-methoxythiobenzoyl)aniline (14).** Prepared as above, from 2,6-dimethyl-*N*-(4-methoxybenzoyl)aniline and Lawesson's reagent, this aniline (87%) crystallized from methanol as yellow prisms, mp 158–160 °C (Found: $M^+$, 271. $C_{16}H_{17}NOS$ requires M, 271). $^1$H NMR δ 3.80 (3H, s, OMe), 2.20 (6H, s, 2 × Me). $\lambda_{max}$ 212, 275 nm.

**General Method for the Jacobson Synthesis of Methoxy-Substituted 2-Phenylbenzothiazoles 9.** Substituted thiobenzanilides 8 (0.01 M) were wetted with a little ethanol, and 30% aqueous sodium hydroxide solution (8 mol equiv) was added.

The mixture was diluted with water to provide a final solution/suspension of 10% aqueous sodium hydroxide. Aliquots of this mixture (1 mL) were added at 1-min intervals to a stirred solution of potassium ferricyanide (4 mol equiv) in water at 80–90 °C. The reaction mixture was heated for a further 30 min and then allowed to cool. Products (9) were collected, washed with water, and crystallized from methanol. Details of yields and physical characteristics are given in Table 2.

**2-(4-Methoxyphenyl)benzothiazole (9b).** 2-Aminothiophenol (5.0 g, 0.04 M) was added to a stirred solution of 4-methoxybenzoyl chloride (6.8 g, 0.04 M) in pyridine (50 mL). The mixture was stirred at 25 °C for 30 min and poured into water (200 mL). The benzothiazole was washed with water and crystallized from methanol to give white needles (8.63 g, 90%), mp 131–132 °C. $^1$H NMR δ 8.01 (1H, s), 7.85 (1H, d), 7.45 (1H, t), 7.34 (1H, t), 6.97 (2H, d), 3.95 (3H, s, OMe). $\lambda_{max}$ 218, 313 nm. The same compound was also prepared by a Jacobsen synthesis (Table 2).

**2-(4-Methoxyphenyl)-5-methoxybenzothiazole (9g).** Cyclization of the thiobenzanilide **8g** (1.50 g, 0.0055 M) by the general method of the Jacobsen synthesis (above) gave a mixture of two products which was separated into 40 fractions by flash column chromatography employing $CHCl_3$ as solvent. Fractions 21–40 were evaporated to yield the 5-methoxybenzothiazole as a white solid (0.34 g, 23%) (Found: C, 66.1; H, 4.8; N, 5.1; $M^+$, 271. $C_{15}H_{13}NO_2S$ requires C, 66.4; H, 4.8; N, 5.2; M, 271). $^1$H NMR δ 3.91 (6H, s, 2 × OMe). $\lambda_{max}$ 226, 298, 335 nm.

**2-(4-Methoxyphenyl)-7-methoxybenzothiazole (9p).** Fractions 1–20 from the above experiment were evaporated to yield the 7-methoxybenzothiazole (0.44 g, 29%) (Found: C, 66.1; H, 4.7; N, 5.1; $M^+$, 271). $^1$H NMR δ 6.95 (6H, s, 2 × OMe). $\lambda_{max}$ 220, 266, 309 nm.

**General Method for the Demethylation of Methoxy-Substituted 2-Phenylbenzothiazoles.** To a stirred suspension of the methoxy-substituted 2-phenylbenzothiazole (0.01 M) in dry dichloromethane (25 mL) under nitrogen at −70 °C was added a 1 M solution of boron tribromide (2 mol equiv for each methoxy group plus a further 3 mol equiv) dropwise over 30–45 min. The mixture was maintained at −70 °C for 1 h and then allowed to warm slowly to 20 °C and stirred overnight. The reaction mixture was recooled to −70 °C and the reaction quenched by the dropwise addition of methanol until no further reaction occurred. The mixture was poured into 8% aqueous sodium hydroxide solution (50 mL). The aqueous phase was separated, acidified with 5 N hydrochloric acid, and extracted three times with a mixture of dichloromethane–methanol (4:1). The combined organic layers were dried (sodium sulfate) and evaporated to yield hydroxy-substituted 2-phenylbenzothiazoles **3**. The crude products were either crystallized from methanol or purified by flash column chromatography employing $CHCl_3$–ethanol (9:1) as solvent. Yields and physical characteristics of the products are listed in Table 3.

**Demethylation of Biochanin A (1b).** Biochanin A (0.5 g) was reacted with a solution of 1 M boron tribromide in dichloromethane (17.6 mL) at −70 °C according to the general method (above). The crude genistein was crystallized from methanol as beige needles (0.33 g), mp 286–287 °C, identical (IR) to an authentic sample.

**2,4,4′-Trihydroxyazobenzene (15a).** 4-Hydroxyaniline (1.0 g) was diazotized at 0 °C in 5 N hydrochloric acid (10 mL) with a solution of sodium nitrite (0.70 g) in water (5 mL). The diazonium solution was added to a solution of resorcinol (1.0 g) in aqueous 10% sodium hydroxide solution (20 mL). The crude azobenzene was collected and crystallized from aqueous methanol to give red microcrystals (0.45 g), mp 241–242 °C.

**Cytotoxicity Assays. MCF-7 Mammary Carcinoma and WiDr Colon Tumor Cells.** Cells were maintained in a continuous logarithmic culture in Dulbecco's medium supplemented with 10% fetal calf serum, penicillin (100 IU/mL), and streptomycin (100 μg/mL). The cells were mildly trypsinized for passage and for use in assays. On day 0, 100 μL of trypsinized tumor cells (1 × 10$^4$/mL) were plated in the wells of 96-well flatbottom microtiter plates. The plates were incubated for 2 days at 37 °C and 5% $CO_2$ in air to allow the cells to adhere and resume exponential growth prior to the addition of drugs.

Test compounds were dissolved in a small volume of DMSO and diluted to the desired concentration with growth medium so that the final concentration of DMSO did not exceed 0.25%. On day 2, 50 μL of the highest drug concentration was added to the wells of column 12 and from there serially diluted 3-fold to column 1 by serial transfer of 50 μL using an 8-channel micropipette. The final volume of column 1 was adjusted to 100 μL. No additions were made to the wells of rows A and B, which served as controls. The plates were further incubated for 5 days at 37 °C and 5% $CO_2$ in air. Each compound was tested in duplicate.

On day 7, the test was terminated by the addition of 100 μL of saline containing 0.002% w/v propidium iodide (Sigma), 0.3% drawing ink (Staedtler marsmatic 745), and 0.5% Triton X-100. The plates were kept at 4 °C overnight before reading on an inverted microscope equipped with an automated scanning stage. Fluorescence intensity was measured in arbitrary units by a photomultiplier. An HP-87 computer controlled the movement of the stage and collected and processed the data from the multiplier.

For each drug substance, a dose–response curve was obtained and the $IC_{50}$ value (the drug concentration producing 50% inhibition of cell growth) was calculated.

**3T3 and ANN-1 Cells.** Cells (2 × 10$^4$) were grown in Dulbecco's modified Eagles medium, supplemented with 10% fetal calf serum. Compounds were dissolved/suspended in either DMF or DMSO such that the final concentration of solvent did not exceed 0.5% v/v. Assays were conducted according to a published method.[4]

**Estradiol Receptor-Binding Assay.** Determination of the relative binding affinity for the calf uterine estrogen receptor was by a published procedure.[10]

**Aromatase-Inhibition Assay.** Aromatase was obtained from the microsomal fraction of human placental tissue, and the reagents and conditions used were those previously described.[20]

**EGF Receptor Tyrosine Kinase-Inhibition Assay.** Membranes were prepared from 10$^8$ A431 cells/mL,[21] and the assay was conducted by a published method.[4]

**HN5 Cell Proliferation Assay.** The method used was based on that of Oliver et al.[22] Cells were seeded at an appropriately low density into 96-well sterile microtiter plates in 10% FBS/DMEM and incubated at 37 °C, 5% $CO_2$. After 6 h, when the cells had adhered to the base of the plate, the medium was removed and replaced by test samples in 10% FBS/DMEM containing the inhibitor under test. The cells were maintained in culture at 37 °C and 5% $CO_2$ for 4 days. Medium was changed daily to avoid exhaustion or degradation of medium components and test substance. On the fourth day, the medium was removed and the cells were fixed in formol-buffered saline for a minimum time of 1 h. Fixative was removed from the wells and replaced with 1% w/v methylene blue in 0.01 M borate buffer (pH 8.5). After 30 min, the excess dye was washed from the cells with 0.01 M borate buffer (pH 8.5); 1:1 v/v ethanol/0.1 M HCl was pipetted into each well to elute the dye from the cells, and absorbance at 650 nm was measured in a microplate photometer.

**Inhibition of Mitogenesis in Human Foreskin Fibroblasts.** Low-passage human foreskin fibroblasts were seeded into sterile 96-well microtiter plates at 2.5 × 10$^4$ cells/well in 10% fetal bovine serum/Dulbecco's MEM (10% FBS/DMEM) and incubated at 37 °C and 5% $CO_2$ for 3 days to attain density-arrested confluence. The medium was changed to 1% FBS/DMEM, and the fibroblasts were incubated for a further 48 h to allow for complete depletion of growth factors from the medium. Test samples were added to the wells in 1:1 v/v PBS/DMEM with 12.5 mM Hepes, 1% bovine serum albumin, 4 μg/mL insulin, and 240 μg/mL transferrin. After incubation for 24 h at 37 °C and 5% $CO_2$, $^{125}$IUdR (92.5 kBq/mL, 74 TBq/mmol, 50 μL/well) in DMEM containing 20 μM FUdR was added to the wells for a further 2h. The medium was removed, and the cells were fixed with 5% trichloroacetic acid (TCA) at 4 °C for 10 min. The cells were washed with 5% cold TCA followed by ethanol and left to dry; 100 μL of 0.2 M NaOH was added to each well to solubilize the cells which were then transferred into tubes, and $^{125}$I was counted in a gamma counter.

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