Articles

Hydroxylated Aromatic Inhibitors of HIV-1 Integrase

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Efficient replication of HIV-1 requires integration of a DNA copy of the viral genome into a chromosome of the host cell. Integration is catalyzed by the viral integrase, and we have previously reported that phenolic moieties in compounds such as flavones, caffeic acid phenethyl ester (CAPE, 2), and curcumin confer inhibitory activity against HIV-1 integrase. We now extend these findings by performing a comprehensive structure–activity relationship using CAPE analogues. Approximately 30 compounds have been prepared as HIV integrase inhibitors based on the structural lead provided by CAPE, which has previously been shown to exhibit an IC_{50} value of 7 μM in our integration assay. These analogues were designed to examine specific features of the parent CAPE structure which may be important for activity. Among the features examined for their effects on inhibitory potency were ring substitution, side chain length and composition, and phenyl ring conformational orientation. In an assay which measured the combined effect of two sequential steps, dinucleotide cleavage and strand transfer, several analogues have IC_{50} values for 3′-processing and strand transfer lower than those of CAPE. Inhibition of strand transfer was assayed using both blunt-ended and "precleaved" DNA substrates. Disintegration using an integrase mutant lacking the N-terminal zinc finger and C-terminal DNA-binding domains was also inhibited by these analogues, suggesting that the binding site for these compounds resides in the central catalytic core. Several CAPE analogues were also tested for selective activity against transformed cells. Taken together, these results suggest that the development of novel antiviral agents for the treatment of acquired immune deficiency syndrome can be based upon inhibition of HIV-1 integrase.

HIV integrase is an enzyme which incorporates the double-stranded DNA product resulting from the reverse transcription of viral RNA into a host genome. HIV integrase has been shown to be obligatory for HIV replication.[1–4] This enzyme is not indigenous to the host and therefore represents an attractive target for new anti-HIV agents.[5,6] Development of targeted inhibitors of HIV integrase requires an enzyme-specific in vitro test system. Such a cell-free assay was originally developed on the basic of the two-step base deletion/transesterification mechanism of the integrase enzyme.[7,8] Basically, radiolabeled oligonucleotides are used to measure the process whereby the integrase initially removes two nucleotide bases from the 3′-end of the linear viral DNA ("3′-processing") and then cuts the host DNA and transesterifies its 5′-end to the viral DNA's newly shortened 3′-end ("integration"). A variant of this procedure was devised to measure both the forward reaction ("strand transfer") as well as the reverse reaction ("disintegration"[9]). In this manner, the assay reflects the combined effect of two sequential steps: dinucleotide cleavage and strand transfer. A number of compounds have been identified using these assays which can inhibit cleavage/integration at low micromolar concentrations.[10–20] Many of these inhibitors contain polyhydroxylated aromatic rings, with several also having secondary degrees of unsaturation and a large variety bearing a flavonoid structure. While flavonoid analogues, such as quercetin (**1**), may prove to be therapeutically useful, one disadvantage shared by members of this series is the potential for collateral toxicity due to inhibition of multiple enzyme systems.[15,21] Of note was the finding that caffeic acid phenylethyl ester (CAPE, **2**), which can be viewed as a simplified flavonoid variant[22] and exhibits preferential cytotoxicity toward tumor cells,[23] retained significant inhibitory potency relative to the flavones (for example, quercetin, **1**).[13,15] We therefore undertook studies to develop structure–activity relationship (SAR) data on CAPE-based compounds as integrase inhibitors.

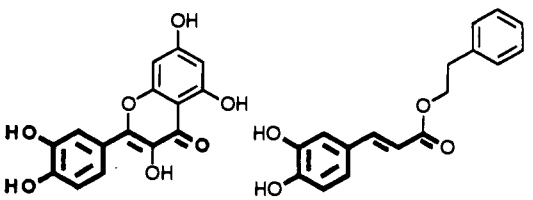

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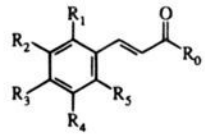
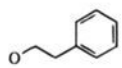
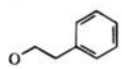
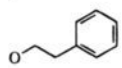
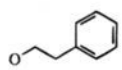
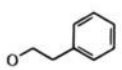
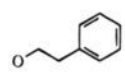
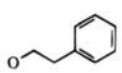
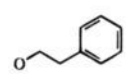
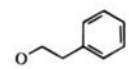
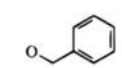
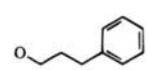
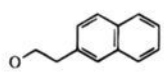
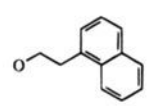
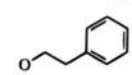
Table 1. Structures of CAPE Analogues with Varying Ring and Side Chain Substituents and Their Associated Integrase IC_{50} Values Determined as Outlined in the Experimental Section[a]



Number	R_0	R_1	R_2	R_3	R_4	R_5	IC_{50}(μM)
2		H	OH	OH	H	H	7
3		H	OH	OCH_3	H	H	60
4		H	OCH_3	OH	H	H	>100
5		H	OCH_3	OCH_3	H	H	>100
6		H	F	F	H	H	>100
7		OH	H	H	OH	H	>100
8		OH	OH	OH	H	H	2
9		H	OH	OH	OH	H	2

Number	R_0	R_1	R_2	R_3	R_4	R_5	IC_{50}(μM)
10		H	OH	OH	H	OH	55
11	OCH_3	H	OH	OH	H	H	>100
12	OCH_2CH_3	H	OH	OH	H	H	>100
13		H	OH	OH	H	H	9
14		H	OH	OH	H	H	8
15		H	OH	OH	H	H	44
16		H	OH	OH	H	H	15
17		H	OH	OH	H	H	11*

[a] An asterisk indicates that the vinyl side chain was reduced to the saturated analogue.

Results and Discussion

In Vitro HIV Integrase SARs. This study has examined the in vitro inhibition of HIV integrase by ca. 30 synthetic compounds. These analogues were designed to examine four aspects of the parent CAPE structure (**2**): (1) ring substituents, (2) ester groups, (3) rotationally constrained variants, and (4) saturated amide analogues.

Effect of Ring Substituents. The number and nature of ring substituents greatly affected potency. As shown in Table 1, replacement of the hydroxyl groups of CAPE (**2**) with either one methyl ether (compound **3** or **4**) or two methyl ethers (**5**) resulted in substantial or complete loss of potency, as did replacement of the hydroxyls by fluorine atoms (compound **6**). Additionally, altering the 3,4-dihydroxy pattern found in CAPE to the 2,5-dihydroxy pattern (compound **7**) of the potent protein-tyrosine kinase (PTK) inhibitor erbstatin resulted in a significant loss of potency. Adding a third hydroxyl to give either the 2,3,4-trihydroxy derivative **8** or the 3,4,5-trihydroxy derivative **9** increased potency approximately 3-fold (Figure 1); however, the 2,4,5-trihydroxy derivative **10** was less potent than the parent CAPE (**2**). The enhancing effect of hydroxyls is consistent with the increase in potency observed when hydroxyls are added to flavone-based integrase inhibitors.[15] The data suggest that *o*-hydroxyl substitution is important. Since *o*-hydroxyl groups are readily oxidized to quinones, oxidation may be involved in the inhibitory mechanism. However, the significant inhibitory potency of monohydroxy compound **3** argues against this. Furthermore, the inactivity of the easily oxidized 2,5-dihydroxy **7** supports the idea that factors other than quinone formation are at work.

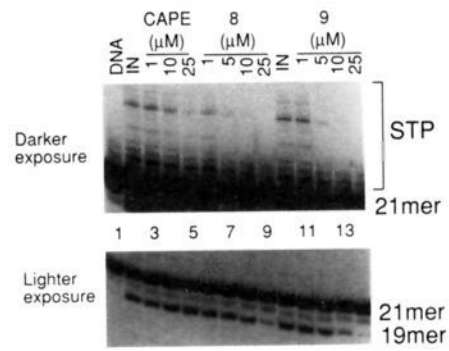


Figure 1. Inhibition of HIV-1 integrase by trihydroxy CAPE analogues **8** and **9**. Autoradiogram showing inhibition of 3′-processing and strand transfer reactions catalyzed by HIV-1 integrase: lane 1, DNA alone; lanes 2 and 10, with integrase; lanes 3–5, in the presence of the indicated concentrations of CAPE (**2**); lanes 6–9, in the presence of the indicated concentrations of **8**; lanes 11–14, in the presence of the indicated concentrations of **9**.

Ester Group. A series of analogues were prepared in which the caffeic acid portion of the molecule (3,4-dihydroxycinnamic acid) was held constant and the phenylethyl side chain was varied. It was found that the aryl ring was required, with the methyl ester **11** and the ethyl ester **12** being inactive. The length of the alkyl spacer was not critical, with from one methylene unit (compound **13**) to three methylene units (compound **14**) between the ester oxygen and the aryl ring having approximately the same potency. The nature of the aryl

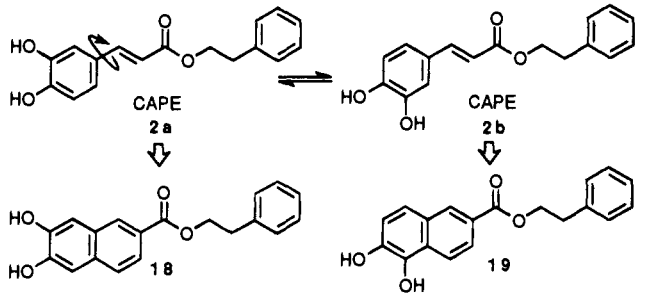


Figure 2. Rotationally constrained CAPE analogues.

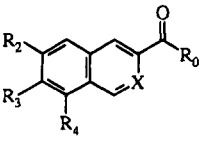
ring was somewhat important, as replacement of the phenyl ring by a naphthyl group resulted in a 5-fold reduction in potency (compound **15**) to a 2-fold reduction in potency (compound **16**). Finally, hydrogenation of the vinylic bond had little effect on potency (compound **17**).

Rotationally Constrained Variants. The dihydroxyphenyl portion of CAPE (**2**) can rotate about the vinylic C−C bond, resulting in two distinct rotational isomers, **2a**,**b** (Figure 2), one of which may display preferential inhibition. Bicyclic analogues **18** and **19**, which differ in the placement of hydroxyl substituents, represent conformationally constrained analogues of the open-chain CAPE rotamers **2a**,**b**, respectively (Figure 2). In the protein-tyrosine kinase (PTK) field, a similar approach using bicyclic nuclei as conformationally constrained mimetics of cinnamic acid-derived PTK inhibitors resulted in analogues, including compound **20**, which displayed greatly differing potencies depending on the hydroxyl substitution pattern.[24,25] By analogy, a significant difference in integrase inhibitory potency could be expected for **18** and **19**. In fact, while the 5,6-dihydroxy derivative **19** was somewhat similar in potency to CAPE, the 6,7-dihydroxy isomer **18** was significantly less potent. Surprisingly, the methyl ester **20** of the 5,6-dihydroxy analogue was equipotent to CAPE. This is in contrast to the results seen in Table 1 with the open-chain CAPE analogues, in which an aryl alkyl ester is required, and may indicate that the bicyclic analogues are working in a somewhat different manner.

A series of isoquinoline analogues were assayed to examine the effect of nitrogen in the bicyclic ring system (Table 2). While methyl ester 6,7-dihydroxyisoquinolinecarboxylate **21** was not active, the corresponding 7,8-dihydroxy isomer **22** was similar to its naphthalene homologue **20** in being nearly equal in potency to CAPE. The greater potency of the 7,8 versus the 6,7-dihydroxy pattern was also seen when aryl amides of the isoquinoline-3-carboxylic acid were prepared. *N*-Benzyl-6,7-dihydroxyisoquinolinecarboxamide **23** was inactive, while the 7,8-dihydroxy isomer **24** exhibited some potency. Finally, increasing the amide chain length of the latter compound provided a slight enhancement of potency (compound **25**).

Saturated Amide Analogues. The importance of the side chain bond and the ester oxygen was examined (Table 3). As pointed out above, the hydrogenated analogue of CAPE (lacking the double bond; compound **17**) retained nearly full potency; however, the corresponding amide analogue proved to be inactive (compound **26**). Full potency could be regained by addition of *o*-hydroxyls to the (phenylethyl)amine ring (compound **27**), while addition of a third hydroxyl (compound **28**) did not increase potency further. Finally, the "reverse amide" analogues **29** and **30** were inactive. The finding that several amide analogues were active is consistent with our recent observations that "tyrphostin" protein kinase inhibitors are also active against HIV-1 integrase.[26] For example, a tyrphostin analogue of compound **27**, in which the original side chain bond present in CAPE is reinserted and a cyano substituent is present

Table 2. Structures of Conformationally Constrained CAPE Analogues and Their Associated Integrase IC_{50} Values Determined as Outlined in the Experimental Section



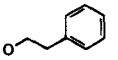
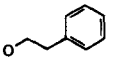
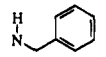
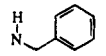
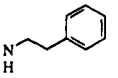
Number	R_0	R_2	R_3	R_4	X	IC_{50}(μM)
18		OH	OH	H	C	68
19		H	OH	OH	C	18
20	OCH_3	H	OH	OH	C	8
21	OCH_3	OH	OH	H	N	>100
22	OCH_3	H	OH	OH	N	14
23		OH	OH	H	N	>100
24		H	OH	OH	N	75
25		H	OH	OH	N	40

Table 3. Structures of CAPE Amide Analogues and Their Associated Integrase IC_{50} Values Determined as Outlined in the Experimental Section

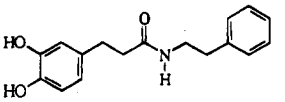
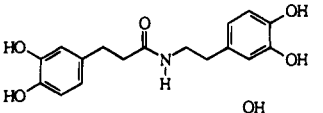
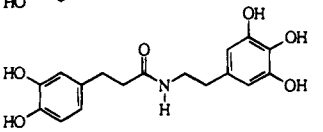
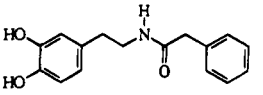
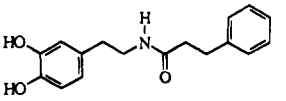
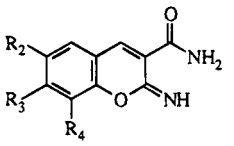
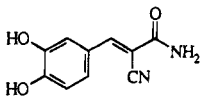
Number	Structure	IC_{50} (μM)
26		>100
27		3
28		4
29		> 100
30		> 100

Table 4. Structures of Miscellaneous CAPE Analogues and Their Associated Integrase IC_{50} Values Determined as Outlined in the Experimental Section



Number	R_2	R_3	R_4	IC_{50}(μM)
31	H	OH	OH	93
32	OH	OH	H	48
33				>100

on the vinylic bond, exhibits IC_{50} values for 3′-processing and strand transfer of 7.5 and 2.7 μM, respectively. These results suggest the possibility that an amide can be substituted for an ester in the central part of analogues without potency loss. However, the finding that *o*-hydroxy sustitution on both phenyl rings is required for activity is not consistent with the ester-based CAPE analogues, where potent inhibition is achieved only with *o*-hydroxy substituents on one ring, and may indicate different modes of binding.

Miscellaneous Structural Variants. Three analogues were also tested which did not fall within the structural categories discussed above. As shown in Table 4, the 7,8-dihydroxyiminochromene **31** and the 6,7-dihydroxyiminochromene **32** showed low potency, while the known PTK inhibitor **33** was inactive.

Site of Action. In an attempt to define the binding site of these CAPE analogues on HIV-1 integrase in more detail, an integrase deletion mutant (IN50-212) lacking the N-terminal zinc finger region and the C-terminal DNA-binding domain[27] was assayed for inhibition by these analogues. This mutant could only be used in the "disintegration" assay because it has been well established that both the amino-(zinc finger) and carboxy- (DNA-binding) terminal domains are required for the HIV-1 3′-processing and strand transfer reactions. CAPE analogues inhibited the disintegration mediated both by the wild-type (IN1-288) and the truncated mutant (IN50-212) enzymes at similar drug concentrations (data not shown). The finding that CAPE analogues are active against the IN50-212 mutant implies that the binding of these analogues to the integrase core region is probably responsible for integrase inhibition. This region is also the target of action of other phenolic derivatives (flavones,[15] curcumin,[20] and biscatechols[18]), copper phenanthroline derivatives,[19] and AZT nucleotides.[16]

Selectivity of Inhibition. As a measure of selectivity against other enzymes (i.e., to gain insights into possible cytotoxicity of these analogues), a deoxyribonuclease I (DNase I) assay was performed with CAPE analogues **8** and **9**. These analogues exhibited no apparent inhibition of DNase I in the same IC_{50} range in which significant inhibition of HIV-1 integrase was observed. In fact, inhibition of DNase I was only observed with **9** at concentrations of 100 μM (data not shown).

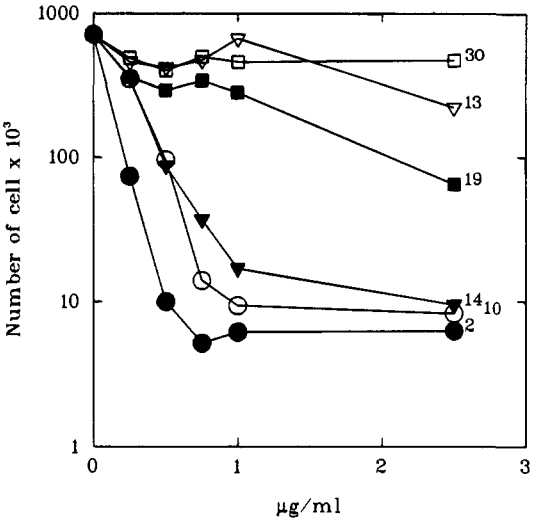


Figure 3. Dose-dependent growth inhibition by analogues of virally transformed rodent fibroblasts (Wt3A) relative to CAPE. Cells were counted after a 72 h incubation in the presence of CAPE or analogues at the indicated concentrations. The number designation for growth-inhibiting analogues and CAPE is at the right.

Cellular Data. All compounds were tested in the NCI in vitro anti-HIV cell screen, with compounds **7**, **13**, and **18** being "moderately active" and compound **26** showing activity just below that required to be so designated. In these four examples, cytotoxicity became significant at doses in slight excess of protective doses, thereby effecting a low therapeutic index. Except for compound **13**, there was no apparent correlation between inhibitory potency in the HIV integrase assay and anti-HIV potency in the cell screen.

CAPE Differentially Affects the Growth of Transformed Cells.[23] In addition to the anti-HIV cell tests, analogues were tested for growth inhibition in comparison to CAPE. In the case of certain human tumor cell lines and cultured adenovirus-transformed rat embryo fibroblasts (Wt3A), addition of CAPE triggers apoptosis, a consequence that is associated with cellular redox change.[28] Activation of this programmed cell death pathway causes Wt3A cells to detach from dishes starting at 4–8 h after addition of a 1 μg/mL CAPE dose. In contrast, similarly treated nontumorigenic parental cells (CREF) sustain merely transient growth inhibition and remain viable. The dose-dependent growth inhibition of analogues was evaluated by measuring Wt3A and CREF cell numbers on triplicate dishes after a 72 h incubation. Cell counts on solvent-treated negative control dishes were typically $(2.5–3.0) \times 10^5$ for Wt3A and $(6–7) \times 10^5$ for CREF cells. CAPE addition to Wt3A cells at 1 μg/mL causes ~50-fold drop in cell numbers at 72 h but only ~5-fold decline in CREF cell counts. Eleven analogues (**5, 6, 8, 10, 13, 14, 18, 19, 29,** and **30**) were tested between 0.5 and 5 μg/mL. Growth inhibitory effects in both cell lines were obtained only in the case of analogues **10, 13, 14, 19,** and **30**. Figure 3 shows the effects of these analogues compared with CAPE in Wt3A cells at doses up to 5 μg/mL. Similar, though less pronounced, effects were observed in CREF cells. The most potent compounds for growth inhibition were **10** and **14**; neither, though, was as

effective as CAPE, itself (**2**). Thus one of the structural elements of the analogues that appears to be required for both the growth effects and inhibition of HIV integrase is diols on the phenyl ring. Both activities may require induction of a redox change. In support of this idea, no inhibition was detected using methylated analogue **5**. However, others have reported compound **5** to be chemopreventive in a rodent carcinogenesis assay.[29] The triol analogue **8**, which has inhibitory activity for HIV integrase, had no effect on cell growth.

Conclusions

The major requirement for potent inhibition of HIV-1 integrase in the present study was two vicinal hydroxyl groups on an aromatic ring. This is consistent with previous SARs of other classes of HIV-1 integrase inhibitors, including flavones,[15] biscatechols,[18] lignans (E. Eich, A. Mazumder, and Y. Pommier, unpublished results), and tyrphostins.[26] In these as well as in the current series, replacement of one or both adjacent hydroxyls with a methoxy group severely reduces potency or renders the compound inactive, while addition of a third adjacent hydroxyl enhances potency. These findings could be consistent with an oxidation–reduction process, which may indicate that inhibition of the enzyme is irreversible. Alternatively, the inhibition may involve chelation of an enzyme-bound divalent metal ion which is thought to be part of the active site.[7,27] Another possibility could be that the hydroxyls function as hydrogen-bond donors, perhaps being required for the aromatic center to be accommodated by a site on the enzyme. The adjacent hydroxyl rule does not apply to all known integrase inhibitors. Of particular interest are certain active diphenols, such as curcumin, in which there are single hydroxyls on each of two phenyl rings separated by a spacer chain.[20] Thus potent inhibition may require two hydroxyls in proximity in space but not necessarily *ortho* to each other on the same ring. This possibility may be pertinent to the high potency of the amide derivative which depended upon the presence of hydroxyl groups on both phenyl rings (**27** relative to **26** and **30**). The corresponding ester, however, only required *o*-hydroxyls on one phenyl ring (**11**), suggesting that subtle conformational factors may come into play.

Experimental Section

Preparation of Radiolabeled DNA Substrates. The following oligonucleotides were purchased from Midland Certified Reagent Co. (Midland, TX):[13,16] AE118, 5′-GTGTGGAAAATCTCTAGCAGT-3′: AE146, 5′-GGACGCCATAGCCCCGGCGCGGTCGCTTTC-3′; AE156, 5′-GTGTGGAAAATCTCTAGCAGGGGCTATGGCGTCC-3′; AE117, 5′-ACTGCTAGAGATTTTCCACAC-3′; AE157, 5′-GAAAGCGACCGCGCC-3′; AE118S, 5′-GTGTGGAAAATCTCTAGCA-3′. These oligonucleotides were purified by HPLC. The AE117 and AE118 oligonucleotides correspond to the U5 end of the HIV LTR. For the 3′-processing and integration assay, AE118 was 5′-end labeled using polynucleotide T4 kinase and [γ-^{32}P]ATP. The kinase was heat-inactivated, and an equimolar amount of AE117 was added. The mixture was heated at 95 °C and allowed to cool slowly to room temperature. The reaction was then run on a G-25 Sephadex quick spin column (Boehringer Mannheim) to separate annealed double-stranded oligonucleotide from unincorporated label. For integration reactions using the precleaved oligonucleotide, AE118S was labeled, annealed, and purified as above.

3′-Processing and Integration Assays. Purified recombinant HIV-1 integrase wild-type was a generous gift of Dr. R. Craigie, Laboratory of Molecular Biology, NIDDK. Reactions were performed as described previously.[13,16] The enzyme was preincubated at a final concentration of 0.25 μM at 30 °C with inhibitor in reaction buffer (50 mM NaCl, 1 mM HEPES, 50 μM EDTA, 50 μM dithiothreitol, 10% glycerol (wt/vol), 7.5 mM $MnCl_2$, 0.1 mg/mL bovine serum albumin, 10 mM 2-mercaptoethanol, 10% dimethyl sulfoxide, and 25 mM MOPS, pH 7.2). After 30 min 0.3 pmol of the labeled cleavage/integration substrate[9] was added, and the incubation was continued for an additional 60 min at 30 °C. The final reaction volume was 16 μL. The reaction was quenched by the addition of an equal volume of Maxam–Gilbert loading dye.

Disintegration Assays. Inhibition of disintegration was performed as above with the "Y" oligonucleotide substrate and an integrase deletion mutant, IN50-212, which lacks the N-terminal zinc finger and C-terminal DNA-binding domains.[13,16] For this assay, AE157 was 5′-end labeled as above. Equimolar amounts of AE117, AE156, and AE146 were added. The mixture was annealed and the reaction run on a G-25 Sephadex quick spin column as above.

Gel Electrophoresis and Quantitation. An aliquot from each reaction was electrophoresed on a denaturing 20% polyacrylamide gel. Gels were dried and subjected to autoradiography using Kodak XAR-2 film or exposed in a Molecular Dynamics PhosphorImager cassette. Gels were analyzed using a Betascope 603 blot analyzer (Betagen, Waltham, MA) or a molecular Dynamics PhosphoroImager instrument (Sunnyvale, CA). Percent inhibition was calculated as described previously.

Cell Growth Assays. Approximately 18 h prior to addition of the analogues, 3×10^4 cells (both Wt3A and CREF) were plated on 35 mm dishes in Ham's F12 medium with 10% fetal calf serum (GibcoBRL, Gaithersburg, MD) and placed at 37 °C in a 5% CO_2 incubator. Experiments used CAPE and its analogues dissolved in 50% EtOH and control treatments of solvent only. Concentrations tested were 0.5, 0.75, 1.0, 2.5, and 5.0 μg/mL. After a 72 h incubation, the cells on all dishes were trypsinized, and triplicate cell numbers were determined for each treatment dose by Coulter counting.

Synthesis. Petroleum ether was of the boiling range 35–60 °C, and removal of solvents was performed by rotary evaporation under reduced pressure. Silica gel chromatography was carried out using TLC grade silica gel (5–25 μm; Aldrich). Preparative HPLCs were conducted using a Vydac preparative C_{18} peptide and protein column with a flow rate of 10 mL/min. Melting points were determined on a Mel Temp II melting point apparatus and are uncorrected. Elemental analyses were obtained from Atlantic Microlab Inc., Norcross, GA, and are within 0.4% of theoretical values unless otherwise indicated. Fast atom bombardment mass spectra (FABMS) were acquired with a VG Analytical 7070E mass spectrometer under the control of a VG 2035 data system. ^{1}H NMR data were obtained on a Bruker AC250 (250 MHz) instrument.

Synthesis of CAPE Analogues. The synthesis of CAPE analogues was achieved by application of literature techniques. Two general approaches, designated "method A" and "method B" were utilized.

Method A: Synthesis of Caffeic Acid β-Phenylethyl Ester (CAPE, 2). A solution of 1.80 g (10.0 mmol) of caffeic acid, 17.9 mL (150 mmol) of β-phenylethyl alcohol, and 100 mg of *p*-toluenesulfonic acid in benzene (100 mL) was stirred overnight at reflux with a Dean–Stark trap. Solvent and excess alcohol were removed by distillation, and the residue was purified by silica gel chromatography (petroleum ether/$CHCl_3$). Product was crystallized (ether/petroleum ether) to provide **2** as snow-white crystals, 1.0 g (35%): mp 128.0 °C (lit.[23] mp 126–128 °C).

β-Phenylethyl 3-Hydroxy-4-methoxycinnamate (3). Reaction of 3-hydroxy-4-methoxycinnamic acid and β-phenylethyl alcohol (method A) provided **3** as cream-colored crystals (21% yield): mp 79.5–80.5 °C; ^{1}H NMR (DMSO-d_6) δ 9.20 (s, 1H), 7.50 (d, 1H, J = 15.7 Hz), 7.36–7.20 (m, 6H), 7.12 (d, 1H, J = 10 Hz), 6.95 (d, 1H, J = 8.3 Hz), 6.32 (d, 1H, J = 15.7 Hz), 4.33 (t, 2H, J = 6.8 Hz), 3.81 (s, 3H), 2.96 (t, 2H, J = 6.8 Hz); FABMS (NBA, −ve) m/z 297 (M − H). Anal. ($C_{18}H_{18}O_4$) C, H.

β-Phenylethyl 4-Hydroxy-3-methoxycinnamate (4). Reaction of 4-hydroxy-3-methoxycinnamic acid and β-phenylethyl alcohol (method A) provided 4 as a colorless syrup (9% yield): ^{1}H NMR (DMSO-d_6) δ 9.62 (s, 1H), 7.54 (d, 1H, J = 15.9 Hz), 7.37–7.17 (m, 6H), 7.11 (d, 1H, J = 8.1 Hz), 6.79 (d, 1H, J = 7.8 Hz), 6.45 (d, 1H, J = 15.9 Hz), 4.34 (t, 2H, J = 6.7 Hz), 3.82 (s, 3H), 2.96 (t, 2H, J = 6.7 Hz). Anal. ($C_{18}H_{18}O_4$) C,H.

β-Phenylethyl 3,4-Dimethoxycinnamate (5). Reaction of 3,4-dimethoxycinnamic acid and β-phenylethyl alcohol was conducted (method A). Removal of solvent and phenylethyl alcohol by distillation yielded a syrup which could be crystallized directly without the aid of chromatographic purification. Product **5** was obtained as snow-white crystals (71% yield): mp 98–99 °C (lit.[30] mp 95–96 °C).

β-Phenylethyl 3,4-Difluoroycinnamate (6). Reaction of 3,4-difluorocinnamic acid and β-phenylethyl alcohol (method A) provided **6** as snow-white crystals (38% yield): mp 53–57 °C; ^{1}H NMR ($CDCl_3$) δ 7.49 (d, 1H, J = 16 Hz), 7.30–7.05 (m, 8H), 6.27 (d, 1H, J = 16 Hz), 4.36 (t, 2H, J = 7.0 Hz), 3.82 (s, 3H), 2.95 (t, 2H, J = 7.0 Hz); FABMS (NBA, +ve) m/z 289 (M + H). Anal. ($C_{17}H_{14}O_2F_2$) C,H.

Method B: Synthesis of β-Phenylethyl 2,5-Dihydroxycinnamate (7). A mixture of 2,5-dihydroxybenzaldehyde (138 mg, 1.0 mmol), 430 mg (0.93 mmol) of (carboxymethyl)-triphenylphosphonium chloride β-phenylethyl ester [mp 162–165 °C dec (lit.[31] mp 148–151 °C)], and powdered anhydrous K_2CO_3 (586 mg, 4.24 mmol) in anhydrous DMF (2 mL) was stirred at ambient temperature overnight. The crude reaction mixture was then partitioned between 0.5 N HCl in brine (50 mL) and ethyl acetate (3 × 50 mL), washed with 0.5 N HCl in brine (50 mL) and with brine (2 × 50 mL), and dried ($MgSO_4$) and solvent removed to yield a dark syrup (577 mg). The crude product was passed down a silica pad using first $CHCl_3$ and then 5% ethyl acetate in $CHCl_3$. The resulting light yellow crystals were recrystallized from ether/petroleum ether (35–60 °C) to provide pure **7** as beige crystals (115 mg, 43% yield): mp 123–125 °C; ^{1}H NMR ($CDCl_3$) δ 7.86 (d, 1H, J = 16 Hz), 7.30–7.17 (m, 5H), 6.88 (d, 1H, J = 2.7 Hz), 6.70 (dd, 1H, J = 2.7, 8.6 Hz), 6.64 (d, 1H, J = 8.6 Hz), 6.44 (d, 1H, J = 16 Hz), 4.36 (t, 2H, J = 7.1 Hz), 2.96 (t, 2H, J = 7.1 Hz); FABMS (NBA, −ve) m/z 283 (M − H). Anal. ($C_{17}H_{16}O_4$) C,H.

β-Phenylethyl 2,3,4-Trihydroxycinnamate (8). Reaction of 2,3,4-trihydroxybenzaldehyde with (carboxymethyl)-triphenylphosphonium chloride β-phenylethyl ester (method B) provided crude product which was purified by multiple passes down a silica pad with final crystallization from ether/petroleum ether, providing pure **8** as beige crystals in 19% yield: mp 144 °C softens, 147–150 °C; ^{1}H NMR (DMSO-d_6) δ 9.77 (s, 1H), 8.60 (s, 1H), 7.78 (d, 1H, J = 16 Hz), 7.36–7.18 (m, 5H), 6.95 (d, 1H, J = 8.5 Hz), 6.37 (d, 1H, J = 16 Hz), 6.36 (d, 1H, J = 8.5 Hz), 4.31 (t, 2H, J = 6.9 Hz), 2.95 (t, 2H, J = 6.9 Hz); FABMS (NBA, −ve) m/z 299 (M − H). Anal. ($C_{17}H_{16}O_5 \cdot {}^1/_4H_2O$) C,H.

β-Phenylethyl 3,4,5-Trihydroxycinnamate (9). Reaction of 3,4,5-trihydroxybenzaldehyde with (carboxymethyl)-triphenylphosphonium chloride β-phenylethyl ester as described above in method B, except that the reaction was run at 80 °C for 5 h, and purification by silica gel chromatography yielded **9** as a light yellow solid in 8% yield: mp 118–124 softens, >160 °C; ^{1}H NMR (DMSO-d_6) δ 7.44 (d, 1H, J = 15.9 Hz), 7.30–7.15 (m, 5H), 6.65 (2, 2H), 6.16 (d, 1H, J = 15.9 Hz), 4.34 (t, 2H, J = 7.0 Hz), 2.94 (t, 2H, J = 7.0 Hz); high-resolution FABMS calcd for $C_{17}H_{15}O_5$ (M − H) 299.092, found 299.056.

β-Phenylethyl 2,4,5-Trihydroxycinnamate (10). Reaction of 3,4,5-trihydroxybenzaldehyde with (carboxymethyl)-triphenylphosphonium chloride β-phenylethyl ester as described above in method B, except that the reaction was run at ambient temperature for 2 h, and purification by multiple silica gel chromatographies yielded a light yellow foam. Trituration with petroleum ether/ether provided **10** as a light yellow solid in 21% yield: mp 146–149 °C; ^{1}H NMR δ 9.62 (s, 1H), 9.52 (s, 1H), 8.45 (s, 1H), 7.75 (d, 1H, J = 16 Hz), 7.37–7.18 (m, 5H), 6.87 (s, 1H), 6.38 (s, 1H), 6.16 (d, 1H, J = 16 Hz), 4.30 (t, 2H, J = 6.8 Hz), 2.95 (t, 2H, J = 6.8 Hz); FABMS (NBA, −ve) m/z 299 (M − H). Anal. ($C_{17}H_{16}O_5 \cdot {}^1/_4H_2O$) C,H.

Methyl 3,4-Dihydroxycinnamate (11). This compound was obtained commercially.

Ethyl 3,4-Dihydroxycinnamate (12). This compound was obtained commercially.

Benzyl 3,4-Dihydroxycinnamate (13). Reaction of caffeic acid and benzyl alcohol (method A) provided **13** as an off-white solid in 42% yield. An analytical sample was obtained by crystallization from ether: mp 151–153 °C; ^{1}H NMR δ 7.54 (d, 1H, J = 15.9 Hz), 7.54–7.31 (m, 5H), 7.07 (d, 1H, J = 2.0 Hz), 7.03 (dd, 1H, J = 2.0, 8.1 Hz), 6.77 (d, 1H, J = 8.1 Hz), 6.34 (d, 1H, J = 15.9 Hz), 5.21 (s, 2H), 3.34 (brs, 2H); FABMS (NBA, −ve) m/z 269 (M − H). Anal. ($C_{16}H_{14}O_4$) C,H.

3-Phenyl-1-propyl 3,4-Dihydroxycinnamate (14). Reaction of caffeic acid and 3-phenyl-1-propanol (method A) provided 14 as an off-white solid in 9% yield: mp 121.5–123.0 °C; ^{1}H NMR δ 7.48 (d, 1H, J = 15.9 Hz), 7.34–7.17 (m, 5H), 7.07 (d, 1H, J = 2.5 Hz), 7.03 (dd, 1H, J = 2.5, 8.1 Hz), 6.78 (d, 1H, J = 8.1 Hz), 6.28 (d, 1H, J = 15.9 Hz), 4.12 (t, 1H, J = 6.5 Hz), 3.35 (brs, 2H), 2.70 (t, 2H, J = 8.1 Hz), 2.02–1.90 (m, 2H); FABMS (NBA, −ve) m/z 297 (M − H). Anal. ($C_{17}H_{18}O_4$) C,H.

2-(2-Naphthyl)ethyl 3,4-Dihydroxycinnamate (15). Reaction of caffeic acid and 2-(2-naphthyl)ethanol as outlined in method A, except that the reaction time was increased to 3 days, provided product as a white solid following chromatography. Trituration with ether gave pure **15** as a snow-white solid in 3% overall yield: mp 174.5–176.5 °C; ^{1}H NMR (DMSO-d_6) δ 9.61 (brs, 1H), 9.14 (brs, 1H), 7.93–7.78 (m, 5H), 7.52–7.42 (m, 3H), 7.04 (s, 1H), 6.99 (d, 1H, J = 8.1 Hz), 6.76 (d, 1H, J = 8.1 Hz), 6.24 (d, 1H, J = 15.9 Hz), 4.43 (t, 2H, J = 6.7 Hz), 3.14 (t, 2H, J = 6.7 Hz); FABMS (NBA, −ve) m/z 333 (M − H). Anal. ($C_{21}H_{18}O_4 \cdot {}^1/_2H_2O$) C,H.

2-(1-Naphthyl)ethyl 3,4-Dihydroxycinnamate (16). Reaction of caffeic acid and 2-(1-naphthyl)ethanol as outlined in method A, except that the reaction time was increased to 6 days, provided product **16** as a snow-white solid in 21% overall yield: mp 165–168 °C; ^{1}H NMR (DMSO-d_6) δ 8.21 (d, 1H, J = 8.1 Hz), 7.97–7.94 (m, 1H), 7.84 (t, 1H, J = 5 Hz), 7.64–7.39 (m, 5H), 7.03 (d, 1H, J = 1.8 Hz), 6.99 (dd, 1H, J = 1.8, 8.1 Hz), 6.23 (d, 1H, J = 15.9 Hz), 4.44 (t, 2H, J = 7.0 Hz), 3.45 (t, 2H, J = 7.0 Hz); FABMS (NBA, −ve) m/z 333 (M − H). Anal. ($C_{21}H_{18}O_4 \cdot {}^1/_4H_2O$) C,H.

β-Phenylethyl 3-(3,4-Dihydroxyphenyl)propanoate (17). A solution of **2** (284 mg, 1.0 mmol) in ethanol (25 mL) was hydrogenated over 10% Pd–C (100 mg) under 40 psi of H_2 in a Parr apparatus (2.5 h). The reaction mixture was filtered through Celite and crystallized from ether/petroleum ether to provide product **17** as off-white crystals (175 mg, 61% yield): mp 72.5–73.5 °C; ^{1}H NMR (DMSO-d_6) δ 8.75 (s, 1H), 8.67 (s, 1H), 7.36–7.18 (m, 5H), 6.61 (d, 1H, J = 8.0 Hz), 6.57 (d, 1H, J = 1.9 Hz), 6.40 (dd, 1H, J = 1.9, 8.0 Hz), 4.21 (t, 2H, J = 6.9 Hz), 2.86 (t, 2H, J = 6.8 Hz), 2.64 (t, 2H, J = 6.9 Hz), 2.49 (t, 2H, J = 6.8 Hz); FABMS (NBA, −ve) m/z 285 (M − H). Anal. ($C_{17}H_{18}O_4$) C,H.

β-Phenylethyl 6,7-Dihydroxy-2-naphthoate (18). A total of 346 mg (1.5 mmol) of 6,7-dimethoxy-2-naphthoyl amide[25] in 6 N HCl (20 mL) was stirred at reflux (24 h) and then cooled and 6,7-dihydroxy-2-naphthoic acid collected as a purple-colored solid (260 mg). A 225 mg (1.10 mmol) portion was esterified with phenylethyl alcohol as described in method A (reaction time 2 days). Chromatographic purification ($CHCl_3$ followed by ethyl acetate) yielded a solid, which was suspended in $CHCl_3$ and collected by filtration to yield **18** as snow-white needles (100 mg, 25% yield overall): mp 175–176 °C; ^{1}H NMR (DMSO-d_6) δ 8.33 (s, 1H), 8.24 (s, 1H), 7.66 (s, 2H), 7.38–7.17 (m, 6H), 4.50 (t, 2H, J = 6.8 Hz), 3.07 (t, 2H, J = 6.8 Hz); FABMS (NBA, −ve) m/z 307 (M − H). Anal. ($C_{19}H_{16}O_4 \cdot {}^1/_4H_2O$) C,H.

β-Phenylethyl 5,6-Dihydroxy-2-naphthoate (19). A total of 240 mg (1.0 mmol) of 5,6-dimethoxy-2-naphthoic acid[25] was heated neat with pyridine hydrochloride (5.0 g) at 180–200 °C under argon (40 min). Excess pyridine hydrochloride was sublimed off under high vacuum and the residue mixed with 1 N HCl (20 mL), giving 5,6-dihydroxy-2-naphthoic acid as a light yellow solid which was collected by filtration (160 mg). A 140 mg (0.7 mmol) portion was reacted with β-phe-

nylethyl alcohol as described for compound **18** and purified by silica gel chromatography ($CHCl_3$) to provide product **19** as a white solid (100 mg, 36% yield overall): mp 164–166 °C; ^{1}H NMR (DMSO-d_6) δ 8.40 (d, 1H, J = 1.6 Hz), 8.07 (d, 1H, J = 8.8 Hz), 7.80 (dd, 1H, J = 1.6, 8.8 Hz), 7.49 (d, 1H, J = 8.6 Hz), 7.38–7.33 (m, 5H), 7.25 (d, 1H, J = 8.6 Hz), 4.51 (t, 2H, J = 6.8 Hz), 3.08 (t, 2H, J = 6.8 Hz); FABMS (NBA, −ve) m/z 307 (M − H). Anal. ($C_{19}H_{16}O_4$) C,H.

Methyl 5,6-Dihydroxy-2-naphthoate (20). This compound was prepared as previously described.[25]

6,7-Dihydroxyisoquinoline-3-carboxylic Acid Methyl Ester Hydrochloride (21). This compound was prepared as previously described.[32]

7,8-Dihydroxyisoquinoline-3-carboxylic Acid Methyl Ester Hydrochloride (22). This compound was prepared as previously described.[32]

***N*-Benzyl-6,7-dihydroxyisoquinoline-3-carboxamide (23).** This compound was prepared as previously described.[24]

***N*-Benzyl-7,8-dihydroxyisoquinoline-3-carboxamide (24).** This compound was prepared as previously described.[24]

7,8-Dihydroxyisoquinoline-3-carboxylic Acid β-Phenylethylamide (25). This compound was prepared as previously described.[24]

***N*-(β-Phenylethyl)-3-(3,4-dihydroxyphenyl)propanamide (26).** To a solution of 1.63 g (9.0 mmol) of 3-(3,4-dihydroxyphenyl)propanoic acid (obtained by hydrogenation of caffeic acid over 10% Pd–C) and pentafluorophenol (1.81 g, 10.8 mmol) in dioxane (40 mL) was added dicyclohexylcarbodiimide (1.85 g, 9.0 mmol) in dioxane (10 mL), and the mixture was stirred at ambient temperature overnight. The mixture was cooled, dicyclohexylurea was removed by filtration, and the solvent was evaporated. The residue was triturated with petroleum ether containing a small amount of ether and 3-(3,4-dihydroxyphenyl)propanoic acid pentafluorophenyl ester collected by filtration as a beige-colored solid (2.84 g, 91% yield). To a solution of 348 mg (1.0 mmol) of this ester in $CHCl_3$ (4 mL) was added β-phenylethylamine (150 μL, 1.2 mmol), and the mixture was stirred overnight. The crude mixture was applied directly to a silica pad and eluted first with 25% ethyl acetate in $CHCl_3$ and then with 100% ethyl acetate. Product **26** was obtained as a light yellow resin (226 mg, 79% yield): ^{1}H NMR ($CDCl_3$) δ 7.26–7.13 (m, 4H), 6.97 (d, 1H, J = 6.5 Hz), 6.71 (d, 1H, J = 8.0 Hz), 6.68 (d, 1H, J = 2 Hz), 6.46 (dd, 1H, J = 2, 8 Hz), 5.65–5.50 (brm, 1H), 3.41–3.32 (m, 2H), 2.72 (t, 2H, J = 7.0 Hz), 2.62 (t, 2H, J = 7 Hz), 2.33 (t, 2H, J = 7 Hz); FABMS (NBA, −ve) m/z 284 (M − H). Anal. ($C_{17}H_{19}O_3 \cdot {}^3/_4H_2O$) C,H,N.

3-(3,4-Dihydroxyphenyl)propanoic Acid [β-(3,4-Dihydroxyphenyl)ethyl]amide (27). To a solution of 348 mg (1.0 mmol) of 3-(3,4-dihydroxyphenyl)propanoic acid pentafluorophenyl ester, prepared as described in the previous reaction, in anhydrous dimethylformamide (2 mL) were added tyramine hydrochloride (227 mg, 1.2 mmol) and triethylamine (209 μL, 1.5 mmol), and the mixture was stirred. After 2 h solvent was removed by distillation under high vacuum and residue purified by silica gel chromatography as described above to yield product **27** as a white foam (338 mg, quantitative yield): ^{1}H NMR (DMSO-d_6) δ 8.75 (s, 1H), 8.72 (s, 1H), 8.16 (s, 1H), 7.84 (t, 1H, J = 5.6 Hz), 6.65–6.57 (m, 4H), 6.44–6.39 (m, 2H), 3.20–3.11 (m, 2H), 2.52–2.46 (m, 2H), 2.28–2.22 (m, 2H); high-resolution FABMS calcd for $C_{17}H_{18}NO_5$ (M − H) 316.1185, found 316.1146. Anal. ($C_{17}H_{19}O_5 \cdot {}^1/_2H_2O$) H,N; C: calcd, 62.14; found, 62.57.

3-(3,4-Dihydroxyphenyl)propanoic Acid [β-(3,4,5-Trihydroxyphenyl)ethyl]amide (28). Using the procedure outlined above for the preparation of compound **27**, except that β-(3,4,5-trihydroxyphenyl)ethylamine hydrochloride was used instead of tyramine hydrochloride, product **28** was obtained in 46% yield as a white foam: ^{1}H NMR (DMSO-d_6) δ 6.62 (d, 1H, J = 8 Hz), 6.57 (d, 1H, J = 2Hz), 6.42 (dd, 1H, J = 2, 8 Hz), 6.10 (s, 2H), 3.19–3.08 (m, 2H), 2.66–2.58 (m, 2H), 2.46–2.36 (m, 2H), 2.30–2.22 (m, 2H); high-resolution FABMS calcd for $C_{17}H_{18}NO_6$ (M − H) 332.1134, found 332.1117.

***N*-[2-(3,4-Dihydroxyphenyl)ethyl]phenylacetamide (29).** To a vigorously stirred mixture of tyramine hydrochloride (948 mg, 5.0 mmol) in aqueous $NaHCO_3$ (1.68 g, 20 mmol in 25 mL of H_2O) and $CHCl_3$ (25 mL) was added phenylacetyl chloride (660 μL, 5.0 mmol) dropwise, and then the mixture was stirred at ambient temperature (1 h). The organic layer was collected and combined with a 25 mL $CHCl_3$ extract of the aqueous layer, the combined organic phases were washed with 1 N HCl (25 mL) and dried ($MgSO_4$), and the solvent was evaporated. The resulting foam was mixed with ethyl acetate and then diluted with ether, and a white solid (225 mg) was removed and discarded. The filtrate was purified by silica gel chromatography using first 25% ethyl acetate in $CHCl_3$ and then 100% ethyl acetate. Product **29** was obtained as a light yellow syrup (362 mg, 27% yield): ^{1}H NMR (DMSO-d_6) δ 7.38–7.24 (m, 5H), 6.37 (d, 1H, J = 8.0 Hz), 6.62 (d, 1H, J = 2 Hz), 6.40 (dd, 1H, J = 2, 8.0 Hz), 5.63 (brt, 1H), 3.14 (dt, 2H, J = 6.5, 13.0 Hz), 2.59 (t, 2H, J = 6.5 Hz); high-resolution FABMS calcd for $C_{16}H_{16}NO_3$ (M − H) 270.1130, found 270.1117. Anal. ($C_{16}H_{17}O_3$) H,N; C: calcd, 69.69; found, 70.11.

***N*-[2-(3,4-Dihydroxyphenyl)ethyl]-3-phenylpropanamide (30).** Using the procedure outline above for the preparation of **29**, except that hydrocinnamoyl chloride was used instead of phenylacetyl chloride, product **30** was obtained as a colorless resin in 13% yield: ^{1}H NMR (DMSO-d_6) δ 7.28–7.05 (m, 5H), 6.72 (d, 1H, J = 8.0 Hz), 6.57 (d, 1H, J = 2 Hz), 6.43 (dd, 1H, J = 2, 8.0 Hz), 5.44 (brm, 1H), 3.36 (dt, 2H, J = 6.5, 13 Hz), 2.86 (t, 2H, J = 7.7 Hz), 2.54 (t, 2H, J = 6.5 Hz), 2.38 (t, 2H, J = 7.7 Hz); FABMS (NBA, −ve) m/z 284 (M − H). Anal. ($C_{17}H_{19}O_3 \cdot {}^1/_2H_2O$) C,H,N.

3-Carbamoyl-7,8-dihydroxy-2-iminochromene (31). This compound was prepared as previously described.[25]

3-Carbamoyl-6,7-dihydroxy-2-iminochromene (32). This compound was prepared as previously described.[25]

3,4-Dihydroxy-α-cyanocinnamide (33). This compound was prepared as previously described.[33]

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