label as HCN through hydrolysis, a compensatory excess of benzyl chloride was employed in the synthesis of the labeled benzyl cyanide to react with the sodium hydroxide and to conserve the active cyanide. The crude active benzyl cyanide was used directly in the next step to avoid losses attendant upon purification, and an excess of ethylene diamine mono-*p*-toluenesulfonate was used in order to conserve the labeled nitrile.

EXPERIMENTAL

Benzyl C^{14}-cyanide. The labeled sodium cyanide[4] (0.10 g.; 2 mmol., specific activity = 20 μc./mg.), containing 0.08 g. (2 mmol.) of sodium hydroxide, was placed in a 50-ml., round bottomed, ground-joint flask and diluted with 0.40 g. (8 mmol.) of inactive sodium cyanide. To this mixture was added 0.5 ml. of water, 2.0 ml. of 95% ethanol, and 1.65 g. (1.51 ml.; 13 mmol.) of benzyl chloride. The mixture was heated under reflux for 4 hr. Anhydrous ether was added and the solution was dried over anhydrous sodium sulfate and filtered. Removal of solvent *in vacuo* left a residue of crude benzyl cyanide.

2-Benzylimidazoline-2-C^{14} hydrochloride. The crude benzyl cyanide from above was treated with 3.01 g. (13 mmol.) of ethylenediamine mono-*p*-toluenesulfonate[2] and the flask was fitted with an air condenser. The mixture was heated at 200° for 1 hr., during which time ammonia gas was evolved, and then allowed to cool to room temperature. The solid was dissolved in 5 ml. of water and the solution was made strongly alkaline with 30% sodium hydroxide solution, precipitating an oil. The oil was taken up in chloroform and this solution was washed well with water. After drying over anhydrous sodium sulfate, the solvent was removed *in vacuo*. The orange gum was dissolved in 1.6 ml. of absolute ethanol and 3.2 ml. of ethyl acetate was added. After saturation with hydrogen chloride gas, 15 ml. of ether was slowly added with shaking. After standing overnight at room temperature, the supernatant liquid was decanted from the reddish brown gum, and this was washed with fresh ether by decantation. It was dissolved in the minimum amount of absolute ethanol and the solution was filtered to remove a small amount of an insoluble contaminant, m.p. 285–300° (uncorr.). The filtrate was heated to boiling and ethyl acetate was slowly added until crystallization had begun. After chilling overnight in the refrigerator, the crystalline material was collected by filtration, washed with a little fresh 6:1 ethyl acetate–absolute ethanol, ethyl acetate, ether, and finally air-dried. The pinkish crystals weighed 0.87 g., m.p. 170–174°. A second crop was obtained by combining mother liquors, adding ether, and crystallizing the resulting gum from absolute ethanol–ethyl acetate. After two additional crystallizations from the same solvent system, an additional 0.15 g. of tan crystals, m.p. 170–172° (uncorr.), was obtained. The combined crops (1.02 g.) were given a final recrystallization from absolute ethanol–ethyl acetate, affording 0.89 g. (45% of theory based on sodium cyanide), m.p. 170–172° (uncorr.) (lit. gives 175°,[2] 168–170°,[5a] 174°[5b]).

Anal. Calcd. for $C_{10}H_{13}N_2Cl$: N, 14.25; Cl, 18.03. Found: N, 14.44; Cl, 18.27.

The specific activity of the product was found[3] to be 1 μc./mg.

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(4) Tracerlab, Inc., 130 High Street, Boston 10, Mass.
(5a) British Patent **460,528; 514,411.**
(5b) S. R. Aspinall, *J. Am. Chem. Soc.*, 61, 3195 (1939).

Identification of Esculetin in Tobacco and in Cigarette Smoke

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In the purification of scopoletin (6-methoxy, 7-hydroxycoumarin) from cigarette smoke and from various tobacco extracts,[1,2] two or more interfering blue fluorescing compounds persisted with the scopoletin through several paper chromatographic developments. The present paper reports our identification of one of these interfering compounds as esculetin (6,7-dihydroxycoumarin).

We have identified esculetin for the first time in the leaves and flowers of oven-dried, greenhouse-grown, One-Sucker tobacco, in the tobacco of representative U. S. cigarettes, and in flue-cured and air-cured tobacco leaf samples. The mainstream smoke from representative U. S. cigarettes was found to contain esculetin in a trace amount.

The esculetin is extremely difficult to separate completely from scopoletin with such solvent systems as 15% acetic acid-water, 60% acetic acid-water; *n*-butyl alcohol–acetic acid–water (6:1:2 v./v.), and *n*-butyl alcohol–benzene-pyridine-water (5:1:3:3 v./v.), but separation on paper chromatograms may be accomplished with the solvent system nitromethane-benzene-water (2:3:5 v./v.).

In addition to its persistence with scopoletin on many paper chromatograms of various tobacco samples, esculetin may be confused on some of these chromatograms with caffeic acid. The R_f values of esculetin and caffeic acid are quite close in a number of solvent systems (Table I), and there is similarity in the bluish white fluorescence of esculetin and of caffeic acid when either is present only in low concentration on the chromatogram. Esculetin, however, behaves differently than does caffeic acid on paper chromatograms still wet with the solvent system *n*-butyl alcohol–benzene-pyridine-water. Under these conditions, esculetin fluoresces a bluish yellow when examined under long wave-length ultraviolet light (3660 Å) whereas the same concentration of caffeic acid exhibits only an extremely weak—practically imperceptible—fluorescence under the same conditions.

EXPERIMENTAL

Esculetin from tobacco flowers. Oven-dried flowers from One-Sucker tobacco plants, *Nicotiana tabacum*, grown in the greenhouse at Argonne National Laboratory, Lemont, Ill., appeared to be richer in esculetin than the leaves and other tobacco samples examined, and were, therefore, used for

(1) C. H. Yang, Y. Nakagawa, and S. H. Wender, *J. Org. Chem.*, **23**, 204 (1958).
(2) C. H. Yang, Y. Nakagawa, and S. H. Wender, *Anal. Chem.*, **30**, 2041 (1958).

many of the early identification studies on esculetin. In a typical experiment, 100 g. of tobacco flowers were extracted in a Soxhlet extractor with two 500-ml. portions of 85% isopropyl alcohol. The combined extracts were concentrated to about 100 ml. *in vacuo*, filtered, and the filtrate acidified to *p*H 2. The filtrate was then subjected to silicic acid column chromatography by a procedure adapted from that of Sondheimer[3] and based on a method described by Bulen *et al.*[4] Fifty-three grams of silicic acid (Mallinckrodt No. 2847) were mixed with the filtrate to produce a thick pasty mixture. This was made into a slurry by addition of 300 ml. of 5% *n*-butyl alcohol–chloroform saturated with 0.5*N* sulfuric acid. The slurry was poured onto silicic acid in a chromatographic column which had been prepared by thorough mixing of 160 g. of silicic acid with 110 ml. of 0.5*N* sulfuric acid, and then adding 1 l. of 5% *n*-butyl alcohol–chloroform saturated with 0.5*N* sulfuric acid. After addition of the slurry to the silicic acid in the column, the 5% *n*-butyl alcohol–chloroform system was used for packing the column. Then for developing and eluting the components of the tobacco flowers, 5%, 15%, 25%, 35%, and 50% *n*-butyl alcohol–chloroform systems saturated with 0.5*N* sulfuric acid were used. Eluate fractions of 500 ml. were collected, concentrated to 100 ml. *in vacuo*, and studied by paper chromatography. The second 500-ml. fraction eluted from the column with the 5% *n*-butyl alcohol system contained the major portion of the esculetin present in the tobacco flowers. This second fraction, after concentration *in vacuo*, was subjected to mass paper chromatography for purification of the esculetin. This eluate was streaked onto four sheets of S & S No. 589, red ribbon, chromatography paper, size 58 × 58 cm., and developed in ethyl acetate–formic acid–water (10:2:3 v./v., upper layer). The bluish white fluorescing zone (approximate R_f = 0.76), containing the esculetin and impurities, was cut out and sewn onto new sheets of the S & S chromatography paper. These were developed in 15% acetic acid–water until the esculetin moved across the bottom thread line. The sheets were then removed from the chamber, dried, and developed in the nitromethane-benzene-water system. The esculetin zone on each paper was cut out, sewn onto still other sheets of the chromatography paper, and developed again in the ethyl acetate–formic acid–water system. The bluish white fluorescing zone, R_f = 0.76, was cut out from each sheet and eluted with 95% ethyl alcohol. The combined alcohol elutions were then studied for proof of identity of esculetin.

The eluted esculetin separated from the tobacco flowers extract was co-chromatographed with the authentic reference esculetin in all the solvent systems mentioned in this paper, both on one- and two-dimensional chromatograms. Typical R_f values are reported in Table I. The eluted esculetin also exhibited the same colors and fluorescence as produced by the authentic reference esculetin when treated with chromogenic sprays (Table II). The phosphotungstic acid and the 10% ammonium hydroxide spray reagents were used in detecting 1-, 2-, and 1,4-hydroxyphenols by Hermann.[5]

TABLE I

R_f VALUES OF ESCULETIN, SCOPOLETIN, AND CAFFEIC ACID

Compound	Solvent Systems[a]					
	(1)	(2)	(3)	(4)	(5)	(6)
Esculetin	0.84	0.84	0.78	0.83	0.09	0.47
Caffeic acid	0.81	0.85	0.80	0.84	0.03	0.42[b]
Scopoletin	0.84	0.82	0.83	0.89	0.80	0.50

[a] Solvent systems: (1) *n*-butyl alcohol–benzene-pyridine-water (5:1:3:3 v./v., upper layer); (2) isopropyl alcohol–pyridine–acetic acid–water (8:8:1:2 v./v.); (3) *n*-butyl alcohol–acetic acid–water (6:1:2 v./v.); (4) ethyl acetate–95% formic acid–water (10:2:3 v./v.); (5) nitromethane-benzene-water (2:3:5 v./v., upper layer); (6) 15% acetic acid–water. [b] A minor spot (R_f = 0.50) was also present.

TABLE II

COLOR[a] REACTIONS OF ESCULETIN, CAFFEIC ACID, AND SCOPOLETIN

Spray Reagent		Compound		
		Esculetin	Caffeic acid	Scopoletin
None, U.V.		Bl-w	bt-Bl	bt-d-Bl
NH_3 vapor, U.V.		bt-Y-Bl	bt-Bl-(e)	bt-Bl
10% NH_4OH and NH_3 vapor, U.V.		bt-Bl-G	bt-Bl	bt-d-Bl
1% alc. $AlCl_3$, U.V.		bt-lt-Bl	bt-Bl	bt-d-Bl
1% alc. $AlCl_3$, NH_3, U.V.		Bl-Y	Bl-W-Y	bt-d-Bl
Phosphotungstic acid and alc. KOH	U.V.	bt-Y	bt-Y	bt-Bl
	V.	ft-Y	ft-Y	N
0.5% $KMnO_4$, aq.	V.	N	bt-Y → Br	N
Diazotized *p*-nitroaniline and Na_2CO_3	V.	N	Y → Br	N

[a] V = visible light, U.V. = ultraviolet light, Bl = blue, Br = brown, G = green, W = white, Y = yellow, bt = bright, d = deep, (e) = enhanced, ft = faint, lt = light, N = no color, → = changing to.

The ultraviolet absorption spectrum of the reference esculetin in 50% ethyl alcohol–water, as determined on a Beckman spectrophotometer, Model DU, exhibited major maxima at 228 and 328 mμ, and a major minimum at 274 mμ. Minor maxima occurred at 254 and 299 mμ, and minor minima at 222, 249, and 310 mμ. The eluted esculetin separated from tobacco flowers gave an identical spectrum, using as a blank a 50% ethyl alcohol–water eluate from the S & S filter paper. The absorption curves of the separated esculetin and the reference esculetin check with the literature.[6]

Preparation of the reference esculetin. Esculetin was prepared from its commercially available glycoside esculin (esculetin-6-glucoside). Ten g. of esculin (Nutritional Biochemical Corp., Cleveland, Ohio) were suspended in 350 ml. of 7% sulfuric acid and heated on a steam bath for 6 hr. The hydrolysate was filtered hot. The fine, needle-like crystals were washed with water and then with ether. The filtrate was cooled overnight. Additional crystals separated and were washed as above. The precipitates were combined, and then the crude esculetin, after decolorization with charcoal, was recrystallized from 95% ethyl alcohol. All commercial samples of esculin that were examined by paper chromatography were found to contain blue fluorescent impurities, and even after hydrolysis and crystallization, the resulting esculetin (4.1 g.) was still not chromatographically pure. Therefore, purification was undertaken by column chromatography of the crystalline esculetin. Two g. were dissolved in methyl alcohol and applied to a column packed with pre-washed Magnesol (Food Machinery and Chemical Corp., N. Y.). The column was developed with ethyl acetate saturated with water. The first 250 ml. of eluate contained impurities and were not used. The subsequent eluates were combined and concentrated *in vacuo*. The resulting precipitate was recrystallized from 95% ethyl alcohol and then sublimed *in vacuo*. This chromato-

(3) E. Sondheimer, *Arch. Biochem. & Biophys.*, **74**, 131 (1958).

(4) W. A. Bulen, J. W. Varner, and R. C. Burrell, *Anal. Chem.*, **24**, 187 (1952).

(5) K. Hermann, *Pharm. Zentralhalle*, **95**, 56 (1956).

(6) R. H. Goodwin and B. M. Pollock, *Arch. Biochem. & Biophys.*, **49**, 1 (1954).

graphically pure esculetin (300 mg.) was used as the reference esculetin, m.p. 270°, with decomposition.[7]

Esculetin in tobacco leaves and in cigarette tobacco. For identification of the relatively smaller amount of esculetin present in tobacco leaves, the procedure described above for tobacco flowers was used. In addition, for some samples, a paper chromatographic procedure was employed which did not involve the preliminary silicic acid chromatography. The first steps of this procedure were the same through the development with the nitromethane-benzene-water system as those already described by Yang *et al.*[2] for the quantitative determination of scopoletin in cigarette tobacco. With the nitromethane system, the scopoletin (R_f = 0.84) moved far ahead of the esculetin (R_f = 0.07). This time, the esculetin zone, still containing another interfering blue fluorescent compound, was cut out and eluted with methyl alcohol. The eluates were streaked on new sheets of S & S paper and developed in 15% acetic acid–water, and then again in the nitromethane system. Each section containing the esculetin was cut out, sewn on a new sheet, developed in the ethyl acetate–formic acid–water system to move the esculetin across the sewing line, and the paper removed and dried. The unfinished chromatogram was then developed again in 15% acetic acid–water to effect the separation of esculetin from the other blue fluorescent compound. Usually esculetin moved sufficiently ahead of the interfering substance at this point to be eluted with methyl alcohol as a chromatographically pure compound and then be identified beyond doubt as esculetin. If not completely separated, the esculetin zone was placed on yet another paper and rechromatographed in the 15% acetic acid–water before making further identification studies.

By one or both of the above procedures, esculetin was identified as being present in a small amount in leaves of Burley tobacco (Kentucky 16), Turkish tobacco (imported and domestic), and flue-cured tobacco (Hicks) from North Carolina.

Because of the low amount of esculetin present relative to that of scopoletin in cigarette tobacco, 8 g. samples were used for these analyses instead of the 2 g. samples used for analysis of scopoletin. Also, Whatman No. 3 MM chromatography paper was used for the first step only in the paper chromatography. The S & S No. 589 red ribbon paper was used for the other paper chromatographic steps. Cigarettes analyzed included Camel, Lucky Strike, Philip Morris, Old Gold Straights, Pall Mall, Viceroy, Winston, and Oasis.

Esculetin in the mainstream smoke from cigarettes. The sampling and smoking of 8 brands of cigarettes for esculetin analysis were similar to those already described for scopoletin by Yang *et al.*[2] The separation, purification, and identification of esculetin from the cigarette smoke condensates were carried out by mass paper chromatography in the same manner described above for esculetin in tobacco leaves. Because esculetin was present only in trace amounts in the smoke, eluates representing smoke from 4 packs of cigarettes had to be combined and concentrated to obtain sufficient esculetin for unambiguous chromatographic studies.

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Addition of Dinitrogen Pentoxide to Stilbene

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The reaction of dinitrogen pentoxide and simple olefins to produce 1,2-nitronitrates has been shown to be a *cis* addition process.[1] As part of this study the reaction of dinitrogen pentoxide and *cis*- and *trans*-stilbene has been investigated. Recently, the *cis* addition of acetyl nitrate to *trans*-stilbene was reported and DL-*threo*-α-acetoxy-α′-nitrobibenzyl was characterized.[2] This compound was a key intermediate in the proof of configuration of the expected products of the dinitrogen pentoxide-stilbene reaction, the α-nitrato-α′-nitrobibenzyls.

Addition of dinitrogen pentoxide to *trans*-stilbene in the presence of tetraethylammonium nitrate[1] produced a mixture of α-nitrato-α′-nitrobibenzyls (81% yield) which was separated into compounds melting at 96° and 165°. The 96° isomer was the predominant product; quantitative infrared analysis of the mixture isolated indicated that it comprised at least 81% of this mixture. Assignment of the *threo* configuration to this α-nitrato-α′-nitrobibenzyl, m.p. 96°, was made on the basis that the same α-hydroxy-α′-nitrobibenzyl[3] which produced the DL-*threo*-α-acetoxy-α′-nitrobibenzyl, m.p. 135°,[2] was converted to the nitronitrate of m.p. 96° on nitration with dinitrogen pentoxide. Thus, the addition of dinitrogen pentoxide to *trans*-stilbene was predominantly a *cis* process.

When the addition of dinitrogen pentoxide to *cis*-stilbene was attempted under the conditions of the *trans*-stilbene addition little reaction occurred and most of the stilbene was recovered.[4] Increasing the reaction time led to a higher yield of nitrated products, but considerable ring nitration apparently occurred. However, the DL-*erythro*-α-nitrato-α′-nitrobibenzyl, isolated in 8.6% yield, comprised a

(1) T. E. Stevens and W. D. Emmons, *J. Am. Chem. Soc.*, **79**, 6008 (1957).

(2) G. Drefahl and H. Crahmer, *Ber.*, **91**, 745 (1958).

(3) The *threo*-α-hydroxy-α-nitrobibenzyl, m.p. 106°, was obtained from the stilbene–dinitrogen tetroxide reaction, the details of which will be reported later. DL-*Erythro*-α-hydroxy-α′-nitrobibenzyl, m.p. 99°, also was obtained from this reaction and was converted to DL-*erythro*-α-acetoxy-α′-nitrobibenzyl, m.p. 116°.

(4) The recovered stilbene was mainly the *trans* form, but isomerization undoubtedly took place during the work-up of the reaction mixture. In the reaction of acetyl nitrate and *cis*-stilbene isomerization proceeded faster than addition.[2]