Peroxidatic Degradation of Flavanones

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Z. Naturforsch. **33 c**, 675–684 (1978); received July 3, 1978

Flavanones, Catabolites, Peroxidases, Degradation, Substitution Pattern

Peroxidases were shown to be responsible for the degradation of flavanones as occuring after application of these substrates to plant cell suspension cultures. — Comparative studies with various flavanones and horseradish peroxidase showed that only 4′-hydroxyflavanones will be catabolized peroxidatically. — Intensive analyses of naringenin (5,7,4′-trihydroxyflavanone) degradation by horseradish peroxidase revealed that very complex reactions with many catabolites are involved. Main degradative pathways comprise a) hydroxilation in the 3′-position, b) elimination of ring B leading to chromones, c) cleavage reactions of the heterocylic ring resulting in phenolic catabolites from ring A and most important d) oxidative destruction of ring A leading to C_6-C_3-, C_6-C_2- and C_6-C_1-units from ring B. — The data are compared with the results of feeding experiments and are discussed with regard to their physiological significance.

Introduction

Flavonoids and various other phenolic compounds are subject to degradation in the producing plants [1, 2]. Catabolic pathways and the enzymes involved are in most cases unknown. Feeding experiments with flavonols, flavanones, aurones and chalcones in plant cell suspension cultures resulted in the observation that the B-rings became liberated as substituted benzoic acids [3–5]. Similar studies with [ring A-^{14}C] flavanones further demonstrated that resorcinol and phloroglucinol rings are very efficiently degraded as indicated by $^{14}CO_2$-production [5].

We now report results of enzymatic studies on flavanone degradation by peroxidases which were shown to be the main flavanone degrading enzymes in plant cell cultures. Our detailed analyses of naringenin (5,7,4′-trihydroxyflavanone) catabolites demonstrate that peroxidatic degradation of flavanones occurs in a very complex manner.

Experimental

Materials

[ring A-^{14}C]naringenin (spec. act. 0.3 mCi/mmol) was obtained from previous studies. All flavanones were commercial products, gifts from various laboratories or specifically synthesized (5–11 in Table I) (unpublished). All solvents and reagents were of analytical grade. Horseradish peroxidase (90 U/mg) was from Roth, Karlsruhe and catalase from Boehringer, Ingelheim.

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Abbreviations: d, doublet; sh, shoulder; UV, ultraviolett; IR, infrared; NMR, nuclear magnetic resonance; MS, mass spectrum; GC, gas chromatography; TLC, thin layer chromatography.

Cell cultures

Growth of cell suspension cultures of soybean and mungbean, extraction and purification of protein from cell cultures, inoculation of labelled compounds, procedures for trapping of $^{14}CO_2$ and labelled material have all previously been published [7, 9].

Experiments with inhibitors

Inhibitory experiments with KCN, catalase and mercaptoethanol on horseradish peroxidase have been published [7].

Standard incubations

The standard assay for measuring peroxidatic degradation of flavanones contained in a final volume of 2.5 ml: 50 µl of 8 mM H_2O_2 and 25 µg horseradish peroxidase with a substrate concentration of 4×10^{-5} M in buffer. 0.2 M citrate-phosphate buffer was used at pH 5.5 and 0.2 M phosphate buffer for measurements at pH 8.0 or 7.5, respectively. Up to about 30% of the buffer could be replaced by ethylene glycol monomethylether or dimethylsulfoxide without severe decrease of enzymatic activity. The reaction was photometrically followed at 322 nm (pH 7.5) or 280 nm (pH 5.5) for at least 20 minutes.

Determination of pH-optima

The procedure of the standard assay was used with the following 0.2 M buffers: formic acid – sodium formate (pH 3.0 – 4.0), citric acid – sodium citrate (pH 4.5 – 5.5), potassiumphosphate – citric acid (pH 6.0 – 7.0), potassiumphosphate buffer (pH 7.5 – 8.0) and glycine – NaOH (pH 8.5 – 10.0). The same standard concentration of naringenin was used at each pH value and all photometric measurements were corrected for the appropriate extinction coefficient at the particular pH-value (s. Fig. 3).

Large-scall incubations

600 mg naringenin dissolved in 360 ml ethylene glycol monoethylether were given into 1200 ml 0.2 M potassium phosphate buffer (pH 7.5) which contained 300 ml 8 mM H_2O_2. The reaction was started with 60 mg horseradish peroxidase in 30 ml buffer, carried on for 30 min at 30°C and terminated by the addition of dil. HCl (pH 4.0). After immediate extraction with ether (5×200 ml, fraction A) the aqueous phase (fraction B) was lyophilized. The dry residue was thoroughly extracted with 200 ml each of a) absolute methanol, b) methanol and c) acetone leaving the bulk of the salts undissolved. Any dissolved inorganic material was eliminated in later stages of purification. The organic solutions were reduced in volume to some 20 ml at room temperature and added to 200 ml of 70% methanol. Methylation was then done for 48 hours in the dark with 200 ml of conc. diazomethane solution (15 g N-nitrosomethylurea). After 24 hours a second batch of CH_2N_2-solution was added.

After drying over Na_2SO_4 fraction A was reduced to 50 ml at room temperature, added to 100 ml of 70% methanol and similarly methylated with CH_2N_2. Excess diazomethane was destroyed with acetic acid and solvent removed under vacuum at room temperature. The methylated products from the various fractions were then subjected to chromatographic separation.

Chromatography

TCL was conducted with the following solvents.
a) on cellulose with

S_1 : 30 percent acetic acid,
S_2 : chloroform: acetic: water = 50:45:5, and
S_3 : isopropanol:water = 8:2;

b) on silica gel with

S_4 : benzene:acetone = 9:1,
S_5 : benzene:ethyl acetate = 55:45,
S_6 : benzene:ethyl acetate = 7:3
S_7 : chloroform:methanol = 100:4
S_8 : chloroform:methanol = 9:1,
S_9 : chloroform:methanol = 7:3,
S_{10}: chloroform,
S_{11}: methanol,
S_{12}: benzene:chloroform = 1:1,
S_{13}: chloroform:methanol = 100:6,
S_{14}: benzene,
S_{15}: benzene:chloroform = 9:7,
S_{16}: benzene:methanol = 9:7 and
S_{17}: benzene:chloroform = 3:7.

Chromatograms were viewed under UV-light (254 and 365 nm) and fluorescing or absorbing bands eluted with $CHCl_3$/MeOH mixtures. All eluates were finally pressed with nitrogen gas through glass filters (G 5) to remove finely dispersed silica gel. Whenever possible catabolites were crystallized from methanol/water mixtures.

Spectroscopic methods

UV-spectra were recorded with a Leitz-Unicam SP 8000 spectrophotometer in methanol. Phenolic compounds were analysed by the addition of diagnostic reagents [10]. IR-spectra were obtained with a Perkin-Elmer-457 Infrared Spectrophotometer with compounds embedded in KBr or as thin films between NaCl discs.

^{1}H-NMR studies were performed with a Bruker WH 90 machine using D_2O or the deuterated forms of chloroform, methanol, acetone and dimethylsulfoxide as solvents. In case of small amounts of material spectra were recorded with pulse and Fourrier transform NMR technique.

Mass spectroscopy was carried out with an Hitachi-Perkin-Elmer mass spectrometer RMV-GD or a Varian SM 16 machine at 70 eV with the ionsource heated to 200°C.

Gaschromatography was conducted with a Hewlett Packard 7610 machine with a tritium EC Detector and a SE 30 column.

GC-MS studies were carried out either with a Varian 111/E1D machine at 80 eV or with a Hewlett-Packard 5750 gaschromatographe (100 m glass capillary column coated with OV 101) attached to a Varian mat CH 7 a mass spectrometer.

Results

Characterization of peroxidases as flavanone degrading enzymes

Upon incubation of naringenin with crude protein preparations from cell suspension cultures of soybean and mungbean flavanone degradation was measured by decrease of absorption at 322 nm (Fig. 1). The spectral changes point to the intermediate formation of other aromatic compounds with the final production of material showing no pronounced UV-absorption. No reaction occurred, however, after ammoniumsulfat precipitation or Sephadex G 25 filtration of the protein extracts, but the reaction could be restored and even intensified by the addition of H_2O_2. Flavanone degradation with or without additional H_2O_2 became completely inhibited by addition of appropriate amounts of catalase, mercaptoethanol or KCN. In presence of low amounts of catalase flavanone destruction could again be obtained with increasing amounts of H_2O_2 which was shown to be an essential cosubstrate.

These and previous [6, 7] data led to similar studies on naringenin degradation with horseradish peroxidase and identical results with respect to H_2O_2 requirements and inhibition by catalase and KCN were obtained.

Enzyme preparations from soybean cell cultures and horseradish peroxidase were further compared for their pH dependence of naringenin degradation in presence of H_2O_2. Identical curves with two maxima around pH 5.5 and 7.5 were observed (Fig. 2). To obtain these curves the pH depending shifts of naringenin absorption were taken into consideration (Fig. 3) and all measurements at either 288 nm or 322 nm were based on the relative absorption coefficients.

Incubations of both enzyme preparations with [ring A-^{14}C] naringenin equally well showed rapid and substantial formation of $^{14}CO_2$ and of polar ether-insoluble material. Chromatographic analysis of both experiments (solvents S_1 to S_3) revealed numerous and in both cases identical compounds. Therefore, naringenin degradation as found in cell cultures or with soybean proxidases which have not yet been purified can well be elucidated by using commercially available horseradish peroxidase.

Substitution pattern of flavanones for peroxidatic degradation

To determine the specificity of peroxidatic flavanone degradation with regard to substitution pattern some 22 flavanones were tested. Due to the sparing solubility of some of the investigated compounds dimethylsulfoxide had to be used to assure solubility. Controls with naringenin showed that up to 30 percent of these solvents did not decisively decrease the rate of peroxidatic flavanone degradation.

In standard incubations with horseradish peroxidase and H_2O_2, degradation of flavanones was measured (Fig. 1) at two pH-values (comp. Fig. 2). The results in Table I indicate that an hydroxyl substituent in position 4′ is an absolute prerequisite for peroxidatic degradation. An additional hydroxyl group in the 7 position facilitates degradation by increasing the reaction rate. Substituents in other position seem to have no essential influence on flavanone degradability.

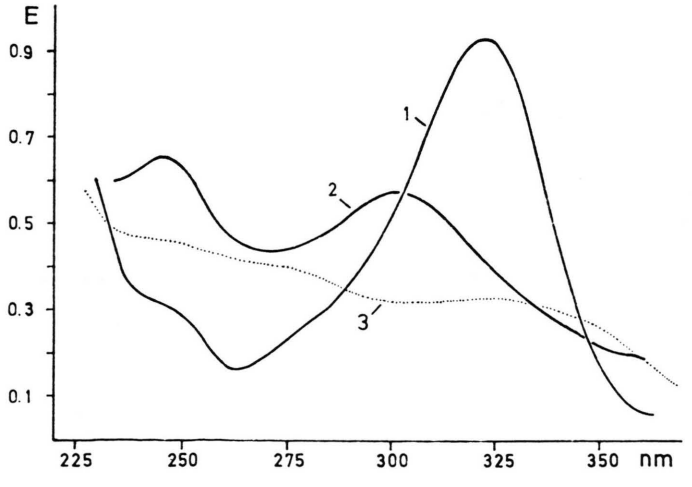


Fig. 1. Change of UV-spectrum of naringenin upon incubation (pH 7.5) with protein preparations from cell suspension cultures or with horseradish peroxidase; 1: spectrum of substrate, 2: after 15 minutes of incubation and 3: after 30 minutes of incubation.

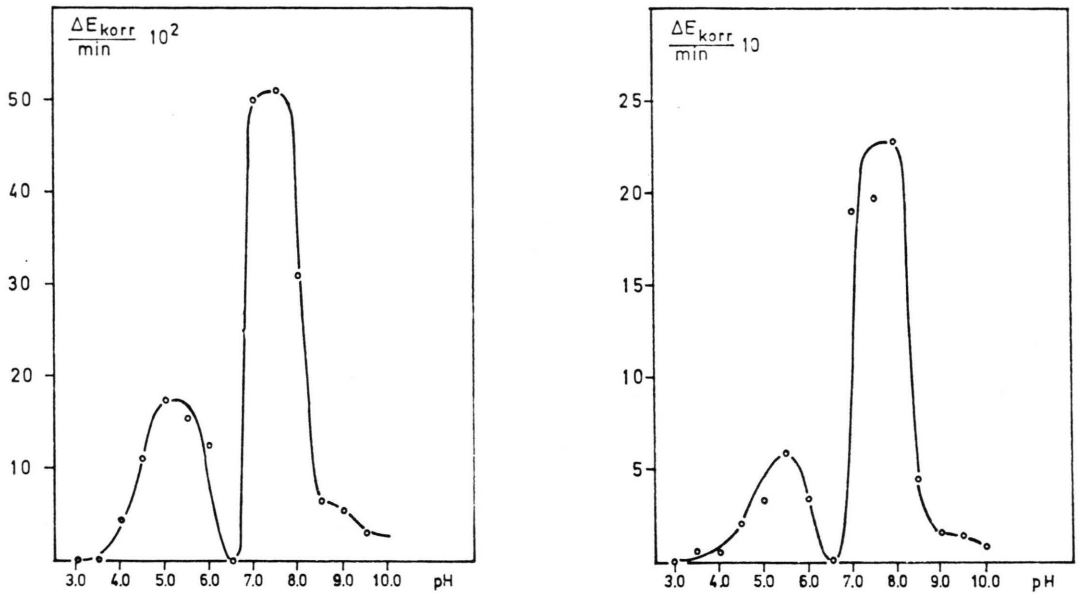


Fig. 2. Dependence of naringenin degradation on pH value with protein from soybean cell cultures (right) and horseradish peroxidase (left).

Isolation of flavanone catabolites

Degradation of naringenin by horseradish peroxidase was carried to a stage as characterized by curve 2 in Fig. 1. Pilot experiments on peroxidatic flavanone degradation had shown that numerous ether-soluble and ether-insoluble catabolites originated and that some of these were rather sensitive to oxidation and polymerisation [8] during chromatographic separation. The products of scaled-up standard incubations with up to 600 mg of substrate (for details see Experimental) were, therefore, permethylated with diazomethan right after extraction into ether (fraction A) or after lyophilisation in case of the water-soluble catabolites (fraction B). The permethylated compounds were separated and purified by repeated thin layer chromatography in system S_3 to S_{17}. Flourescing and UV-absorbing

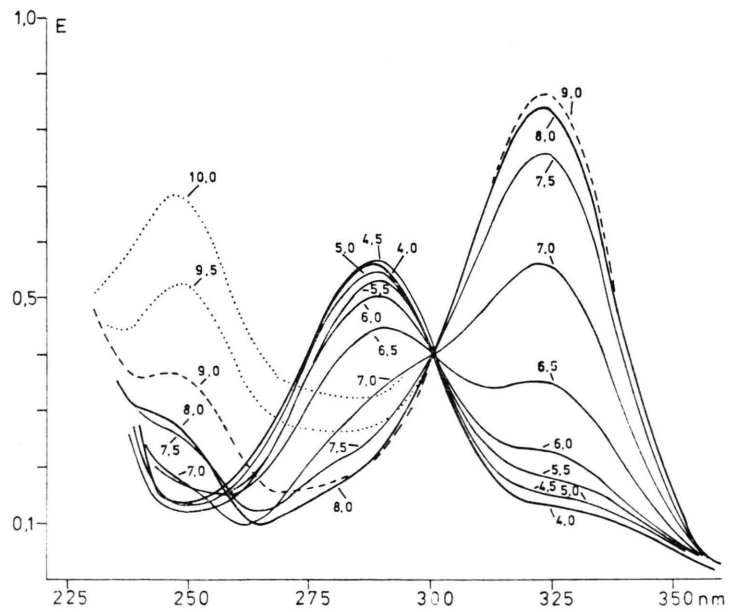
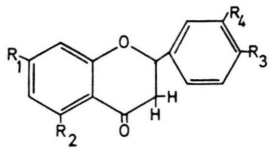


Fig. 3. Shifts in naringenin absorption spectra with pH. Concentration of substrate 0.4 mM.

Table I. Substitution pattern and degradability of flavanones in peroxidase reaction. Standard reactions (see Experimental) were recorded at the long-wave length UV-maximum.



Compound	Substitution pattern				pH value	
	R_1	R_2	R_3	R_4	5.5	8.0
1	OH	H	OH	OCH_3	+	++
2	OCH_3	H	H	H	−	−
3	OH	OH	OH	H	+	++
4	Rhamno-gluco-syl-O-	OH	OH	H	+	+
5	OCH_3	OCH_3	OH	H	+	+*
6	OCH_3	OH	OH	H	+	++*
7	OH	OCH_3	OH	H	+	++*
8	OCH_3	OCH_3	OCH_3	H	−	−
9	OCH_3	OH	OCH_3	H	−	−
10	OH	OCH_3	OCH_3	H	−	−
11	OH	OH	OCH_3	H	−	−
12	OH	H	OH	H	+	+
13	OCH_3	H	OCH_3	H	−	−
14	OH	OH	OH	OH	+	++
15	OH	OH	OH	OCH_3	+	++
16	OH	OH	OCH_3	OH	(+)	(+)
17	gluco-syl-O-	OH	OCH_3	OH	−	(+)
18	OH	OH	OCH_3	OCH_3	−	−
19	OCH_3	H	Cl	H	−	−
20	OCH_3	H	H	OH	(+)	(+)
21	OCH_3	H	H	OCH_3	−	−
22	OCH_3	H	methy-lene-dioxy	−	−	+**

++ Decrease of absorption between 25−90% within 10 minutes.
+ Decrease of absorption between 3−15% within 10 minutes.
(+) Minor changes of UV-maximum.
− No conversion.
* At pH 7.5.
** Reaction due to alcaline hydrolysis of methylene dioxy grouping.

material was preferentially isolated by this procedure. Whenever possible products were obtained by crystallisation. Great care was taken to remove impurities or finely dispersed silica gel by final filtration through very fine glass filters. Frations of some difficult to separate mixtures or compounds which were only obtained in minute amounts were further analysed and purified by gas chromatography followed by mass spectroscopy. Individual compounds were analysed by recording the UV-, IR-, NMR- and mass spectra. Whenever possible catabolites were chromatographically and/or spectroscopically compared with authentic compounds which had been synthesized according to the best method available. Due to the numerous chromatographic steps the yields of the isolated compounds cannot be given.

In some experiments the permethylation procedure was omitted especially in those cases where naringenin methyl ethers (Table I) were investigated as substrates.

Structural elucidation of flavanone catabolites

The catabolites structurally elucidated so far during our studies on peroxidatic flavanone degradation are presented in Fig. 4. The compounds are arranged in a possible reaction sequence though in some cases plausible alternatives exist. The arrangement of compounds in Fig. 4 will, therefore, require further experiments to clearly establish the definite chemical relationship. Furthermore, the compounds are shown as free phenols or acids without the various methyl groups which had been added to facilitate isolation and structural elucidation of some of the sensitive compounds.

The chromone **II** was isolated as the blue fluorescing 5,7-dimethoxyderivative. The UV spectrum (λmax 245, 253 and 283 nm) and the carbonyl absorbtion in the IR spectrum (1640 cm^{-1}) suggested a chromone skeleton. The NMR-spctrum showed two doublets at 6.22 and 7.64 ppm (J = 6.5 Hz) for one H each which are attributable to the protons at C-2 and C-3. The binding sites of two methoxy groups (3.90 and 3.96 ppm, 6 H) were at C-5 and C-7 because the signals of two aromatic protons were observed at 6.39 and 6.47 ppm (J = 2.4 Hz). The structure of **II** was further supported by the fragmentation pattern in the MS spectrum (m/e:M^+ 206(100%) and 205(59), 188 (14), 177(61), 176(15), 175(21), 160(46), 150 (14) and 133(21)).

When the flavanones **5** and **7** (Table I) were subjected to peroxidatic degradation the corresponding chromones were also isolated and their structures similarly determined. Final proof for structural identity was especially obtained by permethylation and chromotographic comparison of products in several solvents because flavanones **3**, **5** and **7** yield the same 5,7-dimethoxy chromone under these conditions.

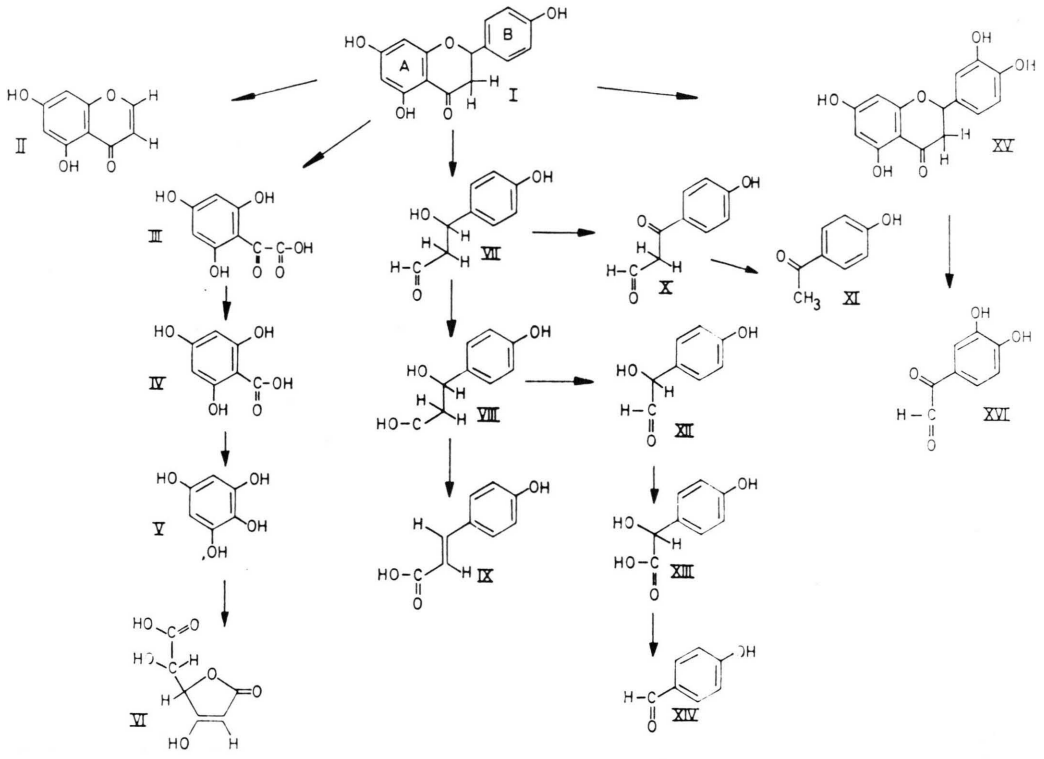


Fig. 4. Catabolities of the degradation of naringenin by horseradish peroxidase. Though most of the compounds were isolated as methylderivatives the methyl groups are not depicted for the sake of clarity.

The α-keto acid **III** was isolated as the methyl ester of the 2,4-dimethylether derivative by gas chromatography. The structure is mainly based on the following analysis of the mass spectrum (*m/e* M^+ 240(36), 209(15, loss of ester O-methyl group), 181(100, loss of CO_2CH_3-function), 151(19, loss of -OCH_2 out of *m/e* 181-fragment), 121 (23, loss of further -OCH_2 out of *m/e* 151-fragment), 125(6, loss of two CO groups out of *m/e* 181 fragment).

Phloroglucinolcarboxylic acid (**IV**) was obtained in form of the 2,4,6-trimethoxy methylester (UV spectrum 250 nm, NMR: methyl groups at 3.80 and 3.76 ppm in a ratio of 1:3, two aromatic protons at 6.20 ppm). Since it was difficult to purify by TLC, the compound was separated from impurities by gaschromatography and its structure determined by a mass spectrum (*m/e* M^+ 226(38), 209(3), 195(100), 167(4), 97(31), 83(36), 69(45), 57(43), 55(40) and 43(48). Authentic material showed an identical mass spectrum.

The phenol **V** was obtained as a trimethyl derivative in very small amounts when some fractions eluted from TLC plates were subjected to GC-MS studies. Especially the mass spectrum (*m/e* M^+ 184(81), 169(100), 141(40), 111(28), 98 (47), 97(63), 83(100) is in agreement with the structure of 2,4,6-trimethoxyphenol.

A compound isolated in small amounts by gas chromatography was tentatively assigned the structure **VI** of an unsaturated lactone-carboxylic acid. This compound can be visualized to arise by ring fision of **V** with subsequent (comp. ref. 36) lactonisation. The UV spectrum (λ max 280 and 275^{sh} nm), the IR spectrum (1470, 1670, 1740^{sh} cm^{-1}) and the NMR-spectrum (2 O-methyl-groups, one at the carboxyl group and the other at the hydroxyl group in the lactone ring) revealed an unsaturated aliphatic compound. This is supported by the mass spectrum where all peaks only differed by 28, 29 or 30 mass units, respectivley. The highest and base peak (*m/e* 171(100)) is odd numbered supporting the assumption of a methylester derivative after the often occurring loss of the methylester group. The molecular peak *m/e* 202 is missing. The other peaks (*m/e* 172(5,7), 143 (25)- 142(9), 113(4), 99(0,6) and 58(94)) are

explained by subsequent elimination of $-CO_2CH_3$, H, HCHO and $-CO_2$ and by cleavage adjacent to the lactone ring. In this case further studies must reveal the validity of the structural suggestion. The aldehyde **VII** was isolated as the dimethylester derivative and structurally elucidated by its spectroscopic data (UV: 276, 281 nm; IR :1730 (carbonyl), 1670, 1650, 1610 and 1500 cm^{-1} (aromatic ring) plus pronounced peaks for aliph. C-H groups; NMR: a *p*-substituted benzene moiety and a well detectable ABX-system at 2.85 and 5.10 ppm; MS:m/e M^+ 194(2,2), 193(6,4); characteristic M-1 peak for aldehydes) 161(6), 151(14), 136 (3,5), 135(10), 121(13) and 108(6)). The spectroscopic difference to the isomeric methylester was especially considered.

The β-hydroxy-dihydro-*p*-coumaric acid (**VIII**) was first isolated as the rather unstable free acid (*RF* 0.8 in S_4; UV λ max 275, 282^{sh} and 325 nm) when naringenin was treated with peoxidase. After methylation, however, a good yield of the *p*-methoxy methyl ester derivative could be obtained. The physical properties (UV spectrum: 268^{sh}, 274 and 281 nm, IR spectrum: 3450 cm^{-1} (hydroxyl), 3005, 2905, 2840 cm^{-1} (aliph. hydrogens), 1730 cm^{-1} (ester carbonyl); NMR: 2 methyl groups at 3.81 and 3.73 ppm, four arom. protons at 7.33/7.29 (d) and 6.91/6.88(d) ppm ($J = 9.5$ Hz), ABX-system at 2.74 ppm ($J = 16.7$ Hz) and 5.1 ppm ($J = 8.8$ and 4.3 Hz). MS after GC separation: *m/e* M^+ 210(2), 192(5), 161(10), 137(31), 136(65), 135(100), 107(28), 92(15), 77(40) and 43(43)) clearly establish the suggested structure. This was further corroborated by the isolation of smaller amounts of the trimethylderivative of **VIII**. In this case no hydroxyl group was detected in the IR-spectrum while 3 methylgroups 3.79, 3.71, 3.69 ppm) and characteristic signals (doublets at 2.79 and 3.08 ppm ($J = 15.5$ Hz) as well as at 5.63 ppm ($J = 5.0/9.1$ Hz)) for the ABX-system were found in the NMR spectrum. The mass spectrum of the latter derivative showed the expected fragmentation scheme with *m/e* 224 as molecular and *m/e* 151 as base peak.

p-Coumaric acid (**IX**) was isolated as the dimethylderivative which upon gas chromatography could be further separated into the cis and trans isomers. The spectroscopic data were matched and found to be identical with those obtained from authentic *p*-methoxycinnamic acid methylester. Compound **X** was found by gas chromatography in the same fraction as **XIV** and it was structurally elucidated by mass spectroscopy (*m/e* M^+ 164(37), 163 (4, M-1 peak indicative of aldehydes), 135 (5, elimination of $-CHO$), 121 (41, removal of $-CH_2CHO$) and 43 (41, signal for CH_2-CHO^+)). The suggested structure should lead to a preferential fragmentation reaction between C_2 and C_3 which is supported by the relative intensity of the fragment ion *m/e* 121.

Compound **XI** was found as the *p*-methoxyderivative during gas chromatography of those TCL fractions which mainly contained the *p*-methoxymethylester of **VIII**. The mass spectrum obtained with the indicated derivative of **XI** (*m/e* 151, M+1, (4), M^+ 150(42), 136(9), 135, M−15 (100), 107, M-43(10), 92, *m/e* 107−15 (16), 77, *m/e* 107−30(23), 64(8), 63(6), 43(9.5)) is characteristic for p-methoxyacetophenone and it was identical in very respect with that of an authentic sample.

The suggested structure of compound **XII** which was isolated by gaschromatography as the dimethylether was especially derived from a careful analysis of the mass spectrum (*m/e* M^+ 180(100), 165(10), 151(29), 137(23), 123(27), 111(45), 109(41), 105(35), 99(51), and 97(82)). The tropyliumion *m/e* 151 is of special importance in this spectrum.

The same tropylium ion *m/e* 151(100) was every pronounced in the mass spectrum of compound **XIII**. The other peaks (*m/e* M^+ 210(89), 195(26), 136(11), 135(12), 108(11) and 107(10)) were also found in the mass spectrum of the authentic methyl ester of the dimethyl derivate of *p*-hydroxymandelic acid. The isolated compound was further found to match the authentic compound in all other properties and it is therefore identical with the trimethyl derivative of **XIII**. The C_6-C_1 structure of **XIV** was found for a compound which appeared in the gas chromatogram of that TCL-fraction from which **V** was also isolated. The structural assignment of the isolated product as anisaldehyde is preferentially based on the identity of the mass spectrum of this substance with both published spectra [11] and that obtained with an authentic sample.

Flavanones **3** and **6** became hydroxilated in the 3′-position because eriodictyol (**XV**), its 7-methylether or the 5,7-dimethylether could all be isolated from the reaction mixtures as such or in permethy-

lated form. Structural identity was determined either by chromatographic comparison (S_1, S_4 and S_6) with authentic samples or by spectroscopic data which were essentially as published [10].

As a likely degradation product of eriodictyol (**XV**) the dihydroxyphenyl substituted aldehyde **XVI** was isolated by gaschromatography as the dimethoxy compound. The mass spectrum possesses the molecular peak M^+ at *m/e* 194(33) and shows the α-ketoaldehyde structure by the characteristic, rather high M-1 peak (*m/e* 193(5), loss of the aldehyde group (M-29 at *m/e* 165(17) with subsequent removal of the α-CO group (165 – 28 (CO) at *m/e* 137(3)). The other peaks were at *m/e* 164 (17), 135(2), 105(3) and 97(20) and are in line with the suggested structure. Except for the methyl groups the NMR spectrum allowed no clear structural assignment due to lack of material.

In addition to compounds **II** – **XVI** shown in Fig. 4 various other catabolites were isolated, but, due to lack of material their structures could not completely be elucidated. Some of them were tentatively identified as acetone dicarboxylic acid and 2-hydroxysuccinic acid semialdehyde. Furthermore traces of the flavonol kaempferol, the flavanonol dihydrokaempferol and of 4,6,4′-trihydroxyaurone were also obtained and they can easily be explained to arise [13 – 15] from the 2′,4′,6′,4-tetrahydroxychalcone by oxidation reactions with H_2O_2. The chalcone itself (not shown in Fig. 4) stems from alcaline conversion of **I** and was isolated in varying amounts.

Discussion

Flavonoids have been characterized as a new class of substrates for peroxidases. In some cases these enzymes have been viewed as being involved in biosynthetic processes [13 – 15] while earlier studies on flavonols [6, 8, 16] and the present investigations on flavanones demonstrate that they may also be associated with catabolic reactions. Fig. 4 clearly demonstrates that peroxidatic degradation of flavanones is a very complex set of reactions with still more compounds occurring than shown in the graph. Due to the isolation procedure employed non-absorbing material and, especially, aliphatic compounds were not discovered in these studies. The compounds structurally elucidated so far allow, however, to explain the formation of CO_2 or constituents of primary metabolism from practically every carbon atom of the flavanone skeleton. This is in agreement with the results of various tracer experiments [1]. It seems, however, feasible that some products may have arisen by purely chemical non-enzymatic reactions either without (**VIII→IX**) or with H_2O_2 (*i.e.* **III→IV** or **VII→VIII**). It must also again be stressed that Fig. 4 only describes apparent chemical relations without proving exact catabolic sequences.

In general, catabolic pathways are arranged in such a way that each individual reaction is catalyzed by a specific enzyme. Flavanone degradation by peroxidases clearly differs from this concept because one enzyme catalyses the complete disintegration of the molecule. The physiological significance of these data must therefore carefully be evaluated [17]. The isolation of $C_6 - C_3$ – (**IX**) and $C_6 - C_1$-compounds (**XIV**) provides enzymatic evidence for identical results of earlier feeding experiments with flavanones, chalcones and dihydrochalcones [1, 3, 5, 18]. The isolation of chromones such as **II** constitutes convincing evidence that these naturally occurring compounds [19 – 22] – unsubstituted at the 2- and 3-positions – may arise by flavonoid degradation and, furthermore, that peroxidases are indeed the responsible enzymes [23]. Taking all evidence together it appears that peroxidases are mainly effective in case of exogenous application of substrate [8, 17] or when the cellular compartmentalisation of phenols and peroxidases is disturbed [21]. Future studies will have to reveal the exact participation of peroxidases in normal flavonoid catabolism under in-vivo conditions.

Peroxidases only degrade 4′-hydroxyflavanones (Table I) and this type of substitution pattern has previously also been determined as prerequisite for peroxidatic destruction of flavonols [24, 25]. Other peroxidatic degradation reactions similarly require a para-hydroxy substituent [7]. The isolated catabolites (Fig. 4) indicate that suitable flavanones are attacked at several positions such as 3′,2,3 and ring A. Cleavage of several carbon bonds thus leads to apparently parallel sets of reactions. Though quantitative analyses of the occurrence of catabolites were not conducted, it appears that the pathways indicated in Fig. 4 are used at comparable rates, because compounds **II**, **VIII**, **IX** and **XV** were found as main products. Formation of $^{14}CO_2$ from [ring A-^{14}C]naringenin

which seems a predominant reaction of **I** and isolated of compounds such as **VI** are further support for the observation [26, 27] that peroxidases may split phenolic rings.

When the naringenin methylethers **5** and **7** were subjected to peroxidatic degradation formation of of the equivalent chromones and hydroxilation in the 3′-position were observed as the prevailing reactions. In case of naringenin 7-0-methylether (**6**) the eriodyctiol (**XV**) 7-0-methylether turned out to be the most important product. These observations indicate that free hydroxyl substituents in ring A are essential for peroxidatic degradation of the phloroglucinol structure and furthermore, that oxidative attack at C-2 of the flavanone skeleton may be regarded as one of the introductionary reactions. The expected intermediate of an 2-hydroxyflavanone was not found during our studies but they were recently isolated as natural products from various plant sources [34]. Future studies must reveal whether these 2-hydroxy compounds are indeed intermediates in flavanone degradation both under natural and *in vitro* conditions.

Meta-hydroxilation (**I** → **XV**) by peroxidases has repeatedly been found with other substrates [28]. This phenolase type of reaction may require the presence of oxidizable cosubstrates (*i. e.* hydroquinones, dihydroxy fumarate) which were, however not added in our studies. It, therefore, seems possible that catabolites of naringenin may have played this role of a donor substance. When naringenin was used as substate comparatively very small amounts of catabolites originating from eriodyctiol **XV** were isolated. This is most probably due to the great sensitivity of the 3,4-dihydroxy structure.

Formation of the hydroxyphloroglucinol **V** may be explained by the previously described [7] oxidative decarboxylation of the *p*-hydroxybenzoic acid **IV**. Formation of **V** may, however, also be explained by assuming oxidative cleavage of the flavanone skeleton between C_4 and ring A. Isolation of catabolites **III**, **IV**, and **V** then indicates that essentially all carbon bonds of the heterocyclic ring of **I** are subject to peroxidatic cleavage. Phloroglucinol carboxylic acid (**IV**) has recently been claimed as a catabolite of flavonol degradation by peroxidases [35] but negative reports are also known [1]. Isolation of such sensitive polyphenols as **V** or **XVI** is presumably due to the methylation procedure employed because studies [7, 8] with larger amounts of free phenols resulted in substantial polymerisation of products. Peroxidases are well known for their capability to oxidatively polymerise phenols [8, 17]. The present studies resulted in only small amounts of polymeric material (~10%) because short time experiments (30 minutes) and early methylation of polyphenolic structures inhibited this side reaction. Despite of these precautions the expected [23] 1,4-dihydroxy substituted aromatic moiety originating from ring B during chromone formation was not found. Further careful analyses of the more volatile catabolites of **I** will have to show whether the postulated mechanism [23] of chromone formation is valid.

Further studies should also inculde the question whether the same set of catabolites is formed when naringenin is subjected to peroxidatic degradation at either pH 5.5 or 7.5 (Fig. 2). Such double pH-optima have previously also been described for tyrosinase [29, 30] and laccase [31, 32] during DOPA-oxidation. In case of **I** they are best explained by assuming that the peroxidase has both different affinities towards and different capability for degradation of the ionized and protonated forms of naringenin which occur depending on the pH value of the solution (Fig. 3). In analogy to other studies [33] one must expect that the pathways of naringenin metabolism (Fig. 4) proceed at very different rates when measured at the two pH-values of Fig. 2. This will further complicate the physiological evaluation of the present data.

Drs. H. Wagner, Munich, and T. J. Mabry, Austin, generously supplied various flavanones. We thank Drs. Bohn, Habersath, Langmann, Löttker, Luftmann, Rücker, Rüterjans, Schulte, Sipply and Stöckelmann, all at Münster, as well as Dr. Gerhards, Bochum, for their help and advice during various spectroscopic studies.

Financial support by Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie is gratefully acknowledged.

Note added in proof.

Recent GC-MS analyses of the permethylether fraction containing catabolites **XI** and **XIV** (see Fig. 4) further resulted in the isolation of an aromatic methoxyderivative ($C_9H_{10}O$) which was found to be *p*-methoxystyrole (**XVII**) especially as indicated by spectral comparison with reference material. The hydroxy derivate of **XVII** can be visualized to arise from **IX** by nonoxidative decarboxylation as known to occur with substituted cinnamic acids (comp. B. J. Finkle, J. C. Lewis, J. W. Corse, and R. E. Lundin, J. Biol. Chem. **237**, 2926 (1962)).

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