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STRUCTURAL DETERMINATION OF COLOURLESS AND YELLOW DIMERS RESULTING FROM (+)-CATECHIN COUPLING CATALYSED BY GRAPE POLYPHENOLOXIDASE

SYLVAIN GUYOT,* JOSEPH VERCAUTEREN† and VÉRONIQUE CHEYNIER

Laboratoire des Polymères et des Techniques Physico-Chimiques, IPV, INRA, 2, Place Viala, 34060 Montpellier, France; †Laboratoire de Pharmacognosie, Université Bordeaux II, 146, rue Leo Saignat, 33076 Bordeaux cedex, France

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Key Word Index—Catechin; polyphenoloxidase; oxidation; catechin dimers; NMR.

Abstract—(+)-Catechin oxidation was carried out in aqueous systems using grape polyphenoloxidase as catalyst. Eight fractions corresponding to the major products formed at pH 3 and 6 were purified using HPLC at the preparative and semi-preparative scale. Structural characterization using UV–visible detection and mass spectrometry indicated that they corresponded to (+)-catechin dimers including two yellow pigments. Mono- and bi-dimensional ^{1}H and ^{13}C NMR analyses provided structural hypotheses for five oxidation products whereas the other three fractions were mixtures of several isomers. Colourless products, with C–C or C–O interflavan linkages, were dehydrodicatechins of the B-type. One of the two pigments corresponds to dehydrodicatechin A, already identified in other oxidation models, and the other pigment is a new structure of the quinone-methide type.

INTRODUCTION

The enzymic oxidation of polyphenols is very important in biochemistry since the subsequent coupling reactions are involved in several biosynthetic pathways including lignin, tannin or melanin formation [1]. In plant derived foods and beverages, these reactions also occur. For example, in the elaboration of fruit juices, wine, beer or cider, coupling of phenols is largely responsible for browning and undesirable hazes [2]. These reactions take place in the presence of oxygen when polyphenoloxidases (PPOs) and their polyphenolic substrates are mixed after bruising, peeling and crushing operations, which lead to rupture of cell structure [3]. The fundamental step in browning is the transformation of *o*-diphenols to the corresponding *o*-quinones [2, 4]. *Ortho*-quinones are highly reactive species involved in different reaction pathways. They are powerful electrophiles which can suffer nucleophilic attack by water [4, 5], other polyphenols [6–8], amino acids, peptides and proteins [3, 9, 10], leading to Michael-type addition products. Owing to their oxidative properties, they are able to oxidize molecules presenting lower redox potentials such as ascorbic acid [5, 11] and other polyphenols which can be converted into other quinonoids [2, 12–14] by coupled oxidation. Under particular conditions, *o*-quinones can also lead to formation of semi-quinone radical anions through a mechanism of reverse disproportionation [15, 16]. These radical species are widely involved in coupling reactions of polyphenols [7, 17].

(+)-Catechin is widely present in numerous fruits, and several studies dealt with its oxidation in the presence of PPO [5, 18, 19]. Most of these investigations were performed using HPLC separation and UV–visible detection. Many condensation products including several yellow compounds were formed, but no identification was provided. Nevertheless, several structures of catechin derivatives obtained by autoxidation [20], chemical oxidation [17] or peroxidase-catalysed oxidation [6] were characterized. For example, in tea model systems based on enzymic oxidation of gallocatechins and their gallates, condensation reactions involved the B rings of the two monomeric units, leading to the formation of theaflavins [21, 22]. Moreover, the prolonged autoxidation or chemical or enzymic oxidation of (+)-catechin led to the formation of polymers resulting from repeated condensation reactions between the A ring of one unit and the B ring of another ('head to tail' polymerization mechanism) [20, 23]. The first isolated products arose from a (+)-catechin oxidized solution obtained by peroxidase catalysis. They were identified as 8-hydroxy-(+)-catechin [24] and catechin dimers referred to as dehydrodicatechin A [6, 25] and dehydrodicatechins B [26]. Dehydrodicatechins B are colourless whereas dehydrodicatechin A is a yellow dimer. Most of these dimers were also formed by chemical oxidation of (+)-catech-

*Present address: Station de Recherche Cidricole et de Biotransformation des Fruits, Légumes et Dérivés, INRA, Domaine de la Motte, BP 29, 35650 Le Rheu, France.

in under alkaline conditions [17] and it was shown that dehydrodicatechins of the B type differ from each other by the nature (C–C or C–O) and the position of their interflavan linkage (IFL) and also by the conformation of the biphenyl moiety [17]. Moreover, they are isomers of some procyanidin dimers of the B type which are natural tannins present in numerous fruits [26]. The major differences concern the position and the nature of their IFL.

Depending on how the oxidation of phenolic compounds is achieved (autoxidation or chemical or enzymic oxidation), the condensation products formed from (+)-catechin may differ. As far as we know, no product resulting from PPO-catalysed oxidation of (+)-catechin has been purified and completely identified. Nevertheless, PPO is responsible for oxidative browning in fruit pomaces and juices, and (+)-catechin is largely represented in fruits. Moreover, (+)-catechin oxidation may lead to the formation of condensation products presenting large structural analogies with natural tannins, which may thus participate in organoleptic characteristics such as astringency, turbidity and colour stability of fruit juices as tannins themselves do.

In a previous study [27], the enzymaic oxidative coupling of (+)-catechin was studied by investigating the formation of the major products under several conditions of incubation pH. HPLC analyses coupled with diode array detection indicated that low pH values favoured the formation of colourless condensation products whereas yellow compounds were formed in larger amounts at higher pH values. In the present work, we describe the purification of the major oxidation products formed at pH 3 and 6. They were then characterized by means of chromatographic, chemical and spectrometric tools in order to elucidate their formations.

RESULTS AND DISCUSSION

(+)-Catechin was oxidized in the presence of a crude grape PPO extract (*Vitis vinifera*, var. Grenache blanc) at pH 3 and 6 for 1 h. The resulting solutions were used to purify the major oxidation products. Although oxidation was performed using the same level of PPO activity at both pH values, the solution oxidized at pH 6 presented a brightly yellow colour whereas the solution oxidized at pH 3 was weakly coloured, indicating some differences for their composition. These solutions were analysed by reversed-phase HPLC coupled with a diode array detector in order to separate the major oxidation products formed and to determine their UV–visible spectrum.

The residual (+)-catechin was eliminated from the solution oxidized at pH 3 using preparative chromatography on Fractogel TSK HW40F. Five major peaks were then collected by semi-preparative HPLC in C18 reversed-phase. The corresponding fractions are noted from I to V in increasing retention time order (Table 1). The three major fractions resulting from (+)-catechin enzymic oxidation at pH 6, noted from VI to VIII (Table 1), were obtained directly by collecting the material corresponding to the peaks in reversed-phase HPLC on the semi-preparative scale. After lyophilization, I–VI fractions corresponded to white amorphous powders, colourless in aqueous or methanolic solutions whereas VII and VIII fractions were orange and yellowish powders, giving, respectively, a bright yellow and a yellow green colouration in water or in methanol. Each fraction was characterized on the basis of its R_t value and UV–visible spectrum by reversed-phase HPLC coupled with a diode array detector and by its R_f value by TLC analysis (Table 1). Results indicated that the eight fractions were 'chromatographically pure'. The UV–visible spectra of colourless fractions were similar to that of (+)-catechin with an absorbance maximum close to 280 nm. Yellow fractions presented several maxima: 280 and 412 nm for VII, and 256, 280 and 385 nm for VIII. Most of the fractions eluted on reversed phase later than (+)-catechin itself (Table 1), suggesting that they may correspond to less polar compounds than (+)-catechin itself. However, two fractions (I and VI) presenting the same R_t on the reversed-phase HPLC column as well as the same R_f on silica gel, eluted before (+)-catechin. Moreover, these two fractions co-eluted with procyanidin B3, but yielded no red colour when submitted to acid hydrolysis, showing that they were not proanthocyanidins. Structural elucidation was then achieved by means of mass and NMR spectrometric analyses. The electrospray mass spectra in the negative mode gave two different molecular ion peaks for the fractions: $[M-H]^-$ at m/z 577 for I–VI and at m/z 575 for VII and VIII. Ion peaks at m/z 599 (for I–VI) and 597 (for VII and VIII), corresponding to $[(M-H)-H+Na]^-$ were also observed. Thus, all fractions corresponded to (+)-catechin dimers. Fractions I–VI, having a molecular weight of 578, presented only one IFL whereas fractions VII and VIII, having a molecular weight of 576, presented a more complex structure (two IFLs and/or one additional unsaturation). The ^{1}H NMR spectra of fractions I, III and IV appeared very crowded and unresolved, suggesting that these fractions were probably mixtures. Consequently, NMR analyses did not give access to the

Table 1. R_f values on TLC silica gel plates [with acetone–toluene–formic acid (4:3:1)], analytical reversed-phase HPLC retention times and absorbance maxima

Fraction or compound	R_f	R_t (min)	λ_{max} (nm)
(+)-Catechin	0.77	12.2	280
I	0.63	10.6	280
II	0.71	14.5	279
III	0.65	20.5	278
IV	0.74	22.6	277
V	0.68	24.8	279
VI	0.63	10.6	280
VII	0.77	26.8	280, 412
VIII	0.88	31.7	256, 280, 385
Procyanidin B3 (or B6)	0.63	10.6	280

complete structure. Nevertheless, the homogeneity of the corresponding mass spectra indicated that these fractions contained isomeric dimers of (+)-catechin which are probably conformational isomers as already observed for (+)-catechin oxidation products [17]. The absence of a fragmentation giving an ion at *m/z* 289 suggests that these dimers presented a biphenyl IFL of the C–C type rather than of the C–O type as observed for products II and V (see discussion below). In the case of fractions II, V, VI, VII and VIII, ^{1}H NMR spectra showed that they corresponded to pure products. ^{13}C, ^{1}H–^{1}H COSY, ^{1}H–^{13}C HMQC [28] (^1J coupling) and HMBC [29] (long-range coupling) experiments were therefore performed, providing structural hypotheses for these five products (in the following discussion, 'product' will be used for fractions containing only a single compound).

Structural elucidation of the colourless dimers II, V and VI and the two yellow dimers VII and VIII

*MS and NMR arguments. (i) Products II and V (Fig. 1, **1** or **2**).* Except for the absence of a phloroglucinol ring AB system (a singlet instead of two doublets), ^{1}H NMR spectra appeared similar to a spectrum of (+)-catechin in which all signals would have been present in duplicate. These observations indicated a C–O IFL between an aromatic hydroxyl group of a (+)-catechin unit and the C-6 or C-8 position of the phloroglucinol ring of the other unit. This argument is supported by the favoured fragmentation (76% for II and 100% for V), giving an ion at *m/z* 289 formed by rupture of the C–O IFL. The major problem was to locate the position of the IFL between the two catechin moieties. The C-8 position of the IFL was confirmed on the basis of the correlations on the HMBC spectrum: the carbon atom C-4aD was easily located because it was the only aromatic non-oxygenated carbon atom (chemical shift close to 100 ppm) which presented correlations with the residual proton of the D ring and also with two H-4 protons. This assignment allowed us to distinguish H-4F protons from H-4C protons. H-2 and H-3 protons of each catechin unit were then identified by observing the two spin systems by the correlations on the ^{1}H–^{1}H COSY spectra which distinguished the protons of each pyran ring. The C-8aD carbon atom was then unambiguously located using an argument already described in the case of a proanthocyanidin dimer [30]: the H-2 proton of the lower unit showed a correlation with a carbon atom, respectively, at 149.2 ppm for II and 149.1 ppm for V, i.e. an oxygenated aromatic carbon atom. This is a ^3J correlation, through the oxygen atom of the F ring, between the H-2F proton and the C-8aD carbon atom which is thus identified. The absence of correlation between this carbon atom and the residual proton of the D ring suggested that this proton is not in the 8-position, which might then be the position of the IFL. However, this argument is insufficient since an absence of correlation is not a proof. The confirmation was obtained by another argument based on HMBC correlations: as we now know where the C-8aD carbon is located, we can also locate the C-5D carbon, which is the only oxygenated aromatic carbon atom (in addition to C-8aD) that shows a correlation with H-4F. This carbon atom also correlates with the residual proton of the D ring so that we can be sure that this proton is located at the 6-position and that, consequently, the lower extremity of the IFL is located at the 8-position. The HMBC experiment did not indicate the position of the upper extremity of the IFL: it was neither possible to observe correlations between carbon atoms of the upper unit and protons of the lower unit nor the inverse because of the large distance due to the ether linkage. However, ES mass spectral data allowed us to exclude eventual positions on the A ring since a fragment ion peak at *m/z* 439 (16% for II and 3% for V), indicating a loss of $C_7H_6O_3$, corresponds to a retro-Diels–Alder rearrangement of the C ring. This fragmentation would not give the same ion if the A ring was substituted by a catechin unit. Thus, the IFL is located on the B ring, but we are not able to distinguish between a C-3′B and a C-4′B substitution since these two carbon atoms could not be completely assignated by NMR. Thus, structures **1** or **2** (Fig. 1) can be proposed for both products II and V.

*(ii) Product VI (Fig. 1, **3** or **4**).* Structure **3** corresponds to dehydrodicatechin B4 already mentioned by Weinges and Huthwelker [26] among the oxidation products of (+)-catechin obtained in the presence of peroxidase. It was also obtained by chemical oxidation of (+)-catechin with potassium ferricyanate [17]. The mass spectrum of product VI differed from those of products II and V by a low intensity of the fragment ion at *m/z* 289 (7%), suggesting that the IFL is probably not of the ether type. The fragment ions *m/z* 439 (21%; *m/z* 461 addition of sodium) and 425 (8%; *m/z* 447 addition of sodium) correspond to the retro-Diels–Alder rearrangements of the C and F rings, respectively. The possibility of both fragmentations in the same molecule indicates that the IFL cannot be located between two phloroglucinol or two catechol rings. Determination of the exact position of the IFL constituted the major problem. The presence, in the ^{1}H NMR spectrum, of two singlets (instead of an ABX system in a non-C-linked B ring) and of one singlet (instead of an AB system in a non-C-linked A ring) indicated a C6′B linkage for the upper position of the IFL and a C-8D or C-6D linkage for the lower position of the IFL, respectively. As already described above for products II and V, HMBC spectra allowed us to locate the C-4aD carbon atom, and the COSY ^{1}H–^{1}H spectrum permitted signal assignment for all protons of the two pyran rings. Unfortunately, the HMBC spectrum did not distinguish unambiguously between a C-8D and a C-6D linkage, as described for products II and V, because the correlation through the oxygen atom of the F ring, allowing the location of the C-8aD carbon atom, was not observed for this compound. According to Weinges *et al.* [31], the C-8D carbon atom presented a higher reactivity compared to that of the C-6D in nucleophilic addition,

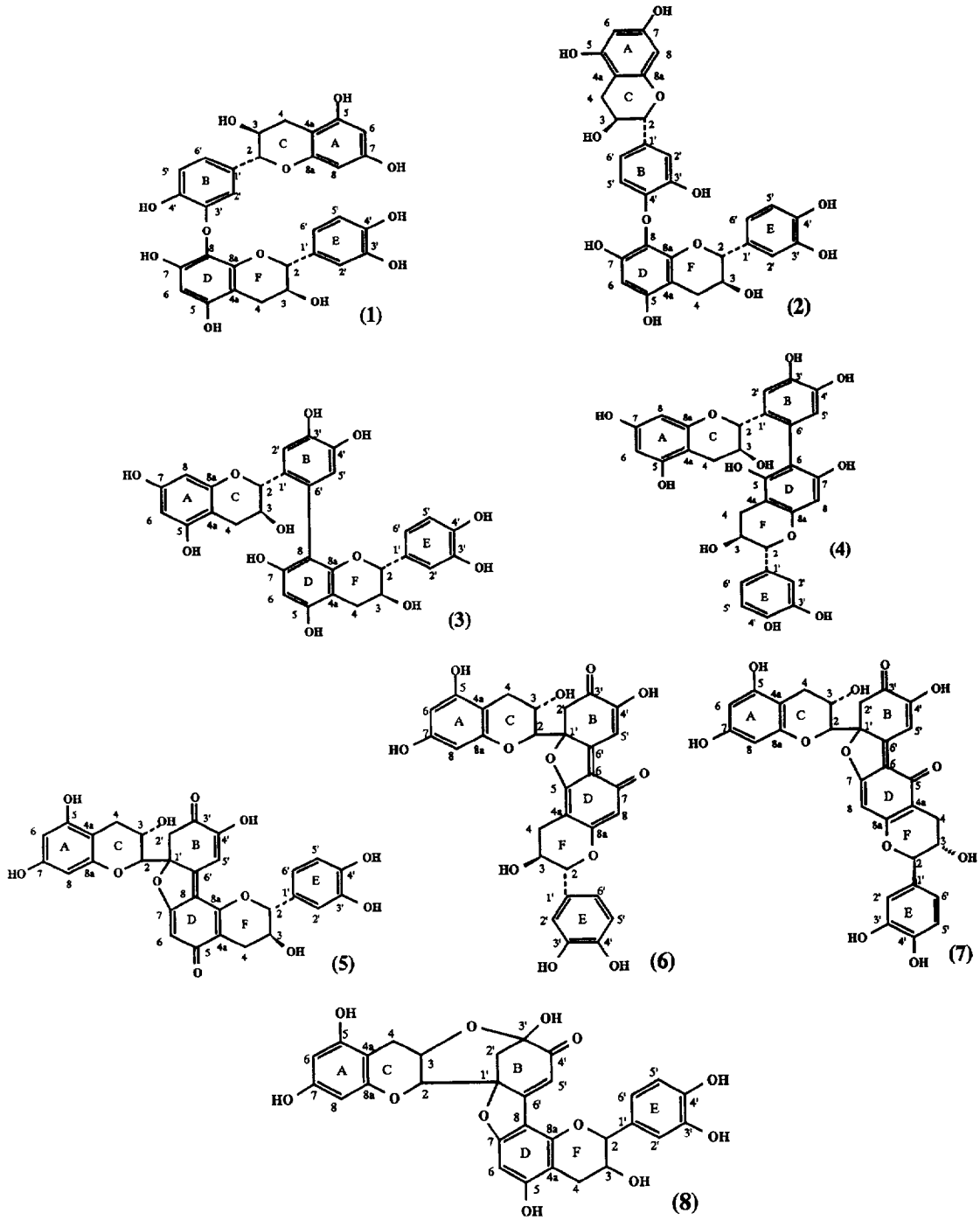

Fig. 1. Structure hypotheses for products II and V (**1** and **2**), VI (**3** or **4**), VII (**5**, **6** or **7**) and VIII (**8**).

which could explain the formation of compound VI. Thus, structure **3** appears to be more probable than a structure presenting a C-6'B–C-6D IFL. However, these arguments do not support structural evidence for the proposed structure **3** (Fig. 1).

(*iii*) *Product VII* (*Fig.* 1, **5**, **6** *or* **7**). Mass analysis revealed a fragment at *m/z* 394 (54%) which was not observed in the mass spectra of compounds II, V and VI. It corresponded to the loss of the benzopyran part ($C_9H_9O_4$) of the upper catechin unit. This favoured fragmentation presumably means that the B ring of compound VII is different from the catechol structure of the colourless dimers. In comparison with those of the other dimers of (+)-catechin described above (structures **1–4**, Fig. 1), the ^{1}H NMR spectrum of fraction VII presented two singular features concerning

the B-ring protons: two singlets (3.10 and 3.64 ppm) with a large coupling constant ($J = 15.6$ Hz) corresponding to the two geminal aliphatic H-2'B protons and a deshielded singlet (6.61 ppm) corresponding to the isolated H-5'B proton involved in a conjugated system. Its ^{13}C NMR spectrum also differed from those of products II, V and VI by the presence of at least four carbons presenting a chemical shift above 160 ppm corresponding to carbons involved in the conjugated ketone systems of B and D rings. C-3'B and C-4'B carbons were located on the basis of their correlations with H-2'B protons in the HMBC spectrum, and their chemical shifts (191.0 and 180.8, respectively) could be explained by their ketonic and enolic nature. Chemical shifts of D-ring carbons and HMBC correlations agree with a quinone-methide structure of this ring. The spectroscopic evidence of the C-8D linkage (rather than C-6D) of the upper unit by means of HMBC correlations, as described above for products II and V, cannot be completely given because of the weak sensitivity of the C-5D carbon atom. Thus, alternative structures **6** and **7** could be proposed for product VII. However, the absence of the correlation between C-8aD carbon and the residual proton of the D-ring is in favour of a C-8D linkage (structure **5**).

(*iv*) *Product VIII* (*Fig.* 1, **8**). As observed for product VII, the mass fragment at m/z 394 (26%) was present, which suggests that the aromatic structure of the B ring may be modified, favouring the loss of the benzopyran moiety of the upper unit. 1D and 2D NMR data permitted us to propose structure **8** (Fig. 1) for product VIII. This structure corresponds to dehydrodicatechin A described earlier by Weinges and Mattauch [25]. All spin systems observed in the ^1H NMR spectrum of product VII were also observed in the spectrum of product VIII. Chemical shifts were almost superimposable except for aliphatic B-rings protons, which were more deshielded ($\Delta\delta = 0.42$ and 1.15 ppm for H-2'αB and H-2'βB, respectively) in the case of product VII than for product VIII, suggesting different structures of the B rings. On the contrary to what was observed for product VII, only one carbon of the B ring resonated above 180 ppm. This observation can be explained by structure **8** (Fig. 1) presenting a hemi-acetal function located at the α'-position of an α–β unsaturated ketonic function. The C-8D involvement in the IFL was deduced from the HMBC correlations, which permitted us to assign the C-8aD and the C-5D carbon atoms as already described for products II and V. The observation of the correlation between the C-5D carbon and the residual proton of the D ring, showing evidence of its 6D position, gave proof of a C-8D IFL. The location at the 5'B position for the ethylenic proton of the B-ring was deduced from its correlation, in the HMBC spectrum, with the C-8D carbon atom. This correlation is not possible in the case of a 2'B position of this proton as proposed earlier for the structure of dehydrodicatechin A [6, 17]. Thus, structure **8** can be proposed for product VIII without ambiguity.

Structure of product VI. Degradation in an aqueous solution at pH 6 indicated the location of the IFL for products VI and VII. A solution of VI at pH 6 was incubated for 24 h, and the medium was analysed before and after incubation by HPLC coupled with a UV–visible diode array detector. The resulting chromatograms at 280, 340 and 420 nm are shown in Fig. 2. Yellow compounds were formed in small amounts after incubation. The two major compounds had the same retention times and UV–visible spectra as products VII and VIII, and are presumably the same. Thus, it seems that products VII and VIII are formed by degradation of product VI at pH 6. As we already know without any ambiguity that product VIII has a C-8D IFL, product VI must also have the same linkage which permitted us to rule out hypothesis **4** (Fig. 1) for this compound. Product VII should also present a C-8D linkage, so that only hypothesis **5** (Fig. 1) can be retained for its structure.

Hypothetical mechanisms for the formation of (+)-catechin oxidation products

The oxidation of *o*-diphenolic compounds in the presence of PPO leads to the formation of the corresponding *o*-quinones [32]. The formation of **3** (Fig. 1), corresponding to the dehydrodicatechin B4, can easily be explained by a nucleophilic attack of the C-8 carbon of a catechin unit on the B ring of the catechin oxidized as a quinone in a Michael-type addition [Fig. 3(a)]. Yellow pigments VII [Fig. 3(b), pathway 2] and VIII [Fig. 3(b), pathway 1] may arise from **3** by two different intramolecular addition mechanisms following the oxidation of the B ring either enzymatically or by a coupled oxidation involving the (+)-catechin quinone as an oxidant. The formation of O-3'B–C–8D and O-4'B–C-8D linkages, observed for **1** and **2** (Fig. 1), respectively, may result from a coupling reaction involving a semi-quinone radical [Fig. 3(c)]. The radical mechanism of coupling may be explained by a homolytic aromatic substitution postulated by McDonald and Hamilton [7] as an alternative mechanism to the more classical homolytic coupling of two radicals. Such a mechanism should prevail especially when each radical produced is surrounded by a much higher concentration of unoxidized phenol molecules, which was the case under our experimental conditions. The formation of B-ring radical species may result from reverse disproportionation reactions [Fig. 3(d)] since the incubation conditions used [high excess of (+)-catechin compared to (+)-catechin quinone] favour these reactions [16].

In our previous study of the influence of pH on the PPO-catalysed oxidation of (+)-catechin [27], it was shown that, for a constant level of PPO activity, the formation of **1** and **2** was favoured by low pH values whereas the formation of **3** and of the two yellow pigments **5** and **8** was favoured by higher pH. These observations are in agreement with the above proposed mechanisms since higher pH values, by increasing the nucleophilic character of (+)-catechin (higher propor-

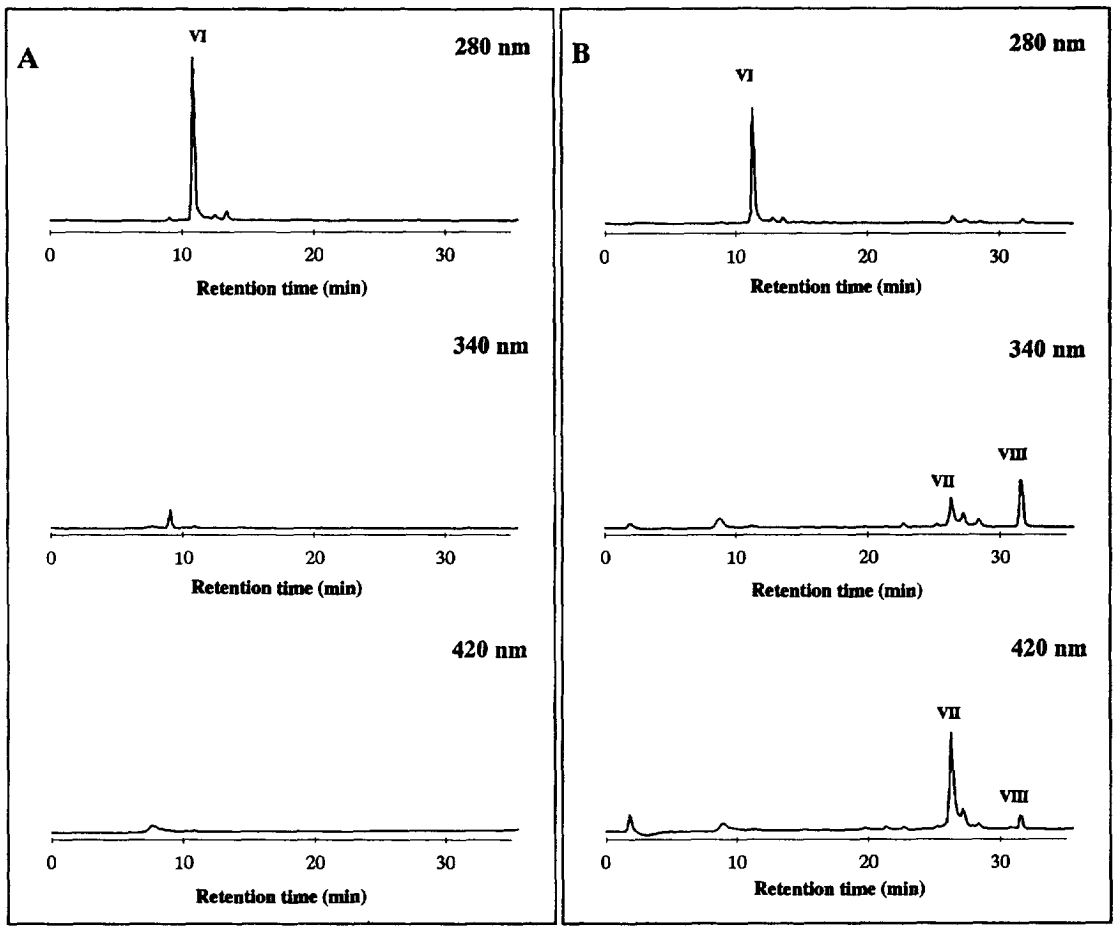

Fig. 2. Reversed-phase HPLC chromatograms of an aqueous solution of product VI at pH 6 before (A) and after (B) incubation for 24 h at 30°.

tion of phenolate anions), should favour the formation of **3** via Michael addition. Consequently, pigments **5** and **8**, deriving from **3**, are also formed in larger amounts when the pH increases. Inversely, lower pH values should impede nucleophilic addition and, at the same time, are expected to increase the reactivity of semi-quinone radicals [15] involved in the mechanism leading to the formation of **1** and **2**.

In conclusion, our results show that oxidation of (+)-catechin in the presence of grape PPO leads to the formation of several dimers which have mostly been also observed in other oxidative conditions (chemical oxidation or oxidation in presence of peroxidase). Colourless dimers present only one C–C or C–O IFL positioned between the B and D rings. Two yellow compounds correspond to dehydro-dicatechin A and to a structure of the quinone-methide type. Our structural analysis based on 2D NMR spectroscopy confirms previous work [30, 33] on the elucidation of the IFL position of C-6 or C-8 substituted catechins. All identified compounds are isomers of natural procyanidin dimers of the A or the B type.

EXPERIMENTAL

Material. (+)-Catechin was purchased from Sigma. Procyanidin B3 (or B6)* was kindly provided by the 'Station d'Oenologie et de Technologie des Produits Végétaux' (INRA, Narbonne, France).

Crude grape PPO extracts. The PPO extract was prepd as described previously [34].

Oxidation procedure. The incubation media (330 ml) contained (+)-catechin (12 mM) and PPO extract (34 nkat ml^{-1}, activity measured polarographically using 10 mM 4-methylcatechol as substrate, pH 4.6, 25°) in 0.1 M citrate/phosphate buffers at pH 3 or 6. The reaction was started by addition of enzyme extract which was suspended in the same buffer and sonicated for 3 min immediately before use. The incubation was conducted under air agitation for 1 h. The suspensions (pH 3 and pH 6) were immediately centrifuged

*Thiolysis [35] permitted us to be sure of the (+)-catechin nature of the monomeric units but a doubt still existed about C6 or C8 position of the IFL.

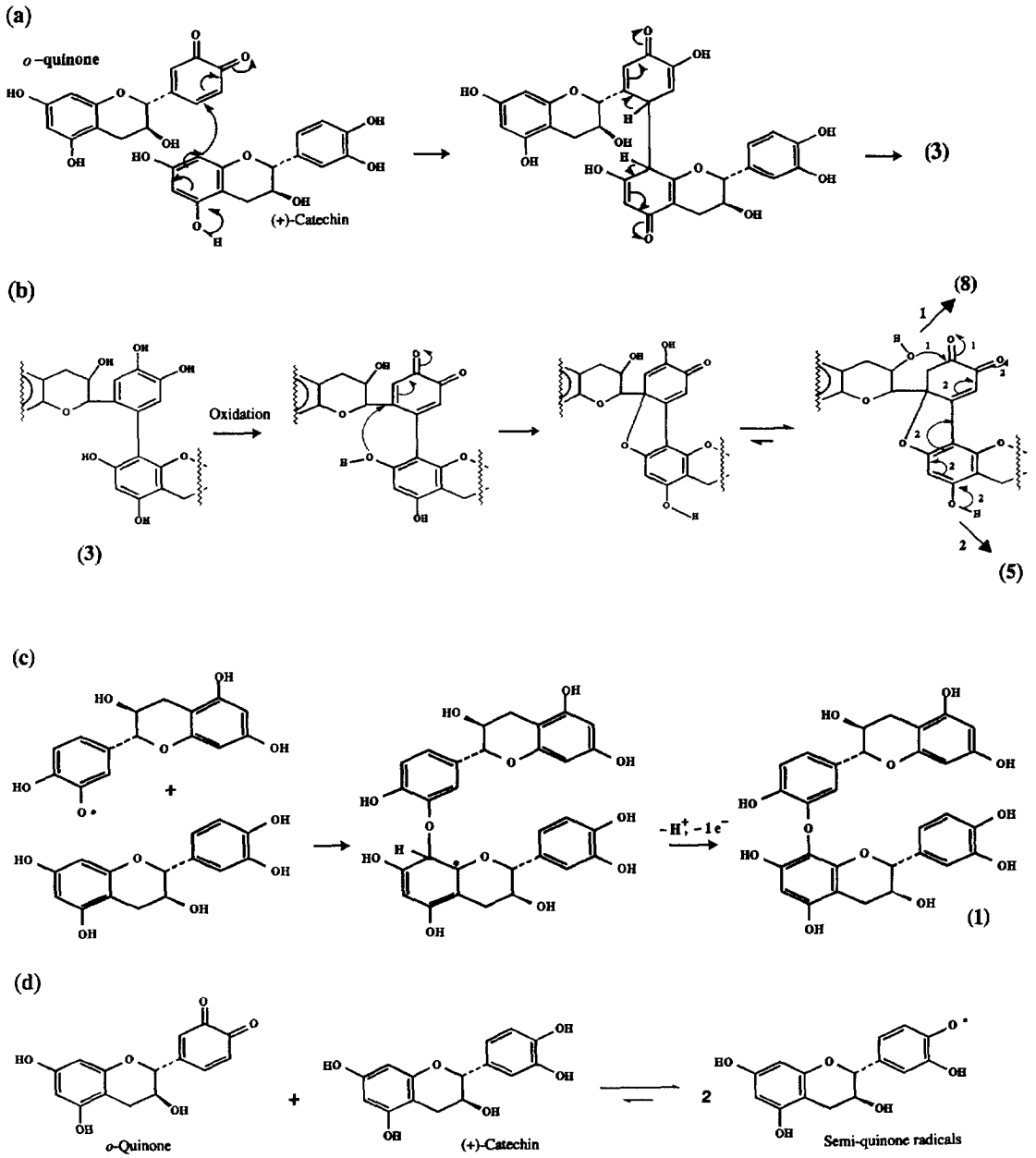

Fig. 3. Mechanisms for the formation of products VI (a), VII and VIII (b), and II and V (c and d).

(4000 *g*) at 4° for 10 min and the supernatants were filtered *in vacuo* through a 0.45 μm membrane filter (HV, Millipore) protected with a glass microfibre prefilter (GF/C, Whatman). The solns were stored at −18° until used.

Purification procedure of oxidation products from soln of (+)-catechin oxidized at pH 3. The soln oxidized at pH 3 was chromatographed using a high pressure prep. chromatographic system including two gradient pumps (M306, head pump 200WTi, Gilson), an injection pump (M306, head pump 10SC, Gilson), a hydraulic compression column (500 × 40 mm i.d., ISA Jobin-Yvon) column, a UV detector (M875-UV, Jasco) set at 280 nm. The column was filled with Fractogel TSK HW-40F (500 ml) pressed under 7 bars and eluted with MeOH (20 ml min^{-1}). Series of injections (40 ml) were performed and two distinct frs were collected each time. The first eluted one corresponded to residual (+)-catechin. The second, containing a mixt. of oxidation products, was evapd and the residue was dispersed in an acidic aq. soln (2.5% HOAc) and freeze-dried. The powder (320 mg) was chromatographed on a semi-prep. HPLC Waters system including a 680 gradient controller, two M510 pumps, a manual U6K injector, a 481 UV detector set at 280 nm and a 730 recorder. The column was a C18 Spherisorb ODS-2 (5 μm packing) (250 × 8 mm i.d.) protected by a guard cartridge packed with same material and heated at 30°. Series of

injections (1.75 ml) of a soln of the powder (2.5 g l^{-1} in 2.5% aq. HOAc) were performed under the following elution conditions: flow rate, 2 ml min^{-1}; solvent A, 2.5% HOAc in H_2O; solvent B, MeCN–solvent A (4:1); linear gradients from 10 to 15% B in 10 min and from 15 to 25% in 10 min followed by washing and reconditioning of the column. Five frs, corresponding to major HPLC peaks, noted from I to V, were collected, evapd and freeze-dried.

Purification procedure of oxidation products from soln of (+)-catechin oxidized at pH 6. The soln oxidized at pH 6 was directly chromatographed using the same semiprep. HPLC system as described above. Series of injections (1.75 ml) were performed under the same elution conditions as described above for sepn of oxidation products obtained at pH 3. Three frs, noted from VI to VIII, corresponding to major HPLC peaks [except that of residual (+)-catechin], were collected, evd and freeze-dried.

Incubation of a soln of fr. VI at pH 6. A 1 g l^{-1} soln of fr. VI in a citrate/phosphate buffer (0.1 M, pH 6) was incubated at 30° under air agitation for 24 h. The medium was analysed before and after incubation by injecting 25 μl on to the analyt. HPLC system described below.

TLC analysis. Frs I to VIII were eluted on silica gel plates (DC-Alufolien Kieselgel 60 F_{254}, Merck) using Me_2CO–toluene–HCO_2H (4:3:1) as eluent. (+)-Catechin and a procyanidin extract of cacao beans [35] were used as refs. Detection was performed by observing the plate under 254 nm light.

Acid hydrolysis. Soln A: $Fe(NH_4)(SO_4)_2$, $12H_2O$, 2% in 2N HCl; sol B: *n*-BuOH–12 N HCl (19:1). 100 μl MeOH (3 g l^{-1}) of each fr. were mixed with 100 μl soln A and 2.5 ml soln B. The mixt. was incubated at 95° for 45 min.

HPLC system for analytical experiments. HPLC apparatus: Kontron Instruments (Milan, Italy) system including a 460 autosampler, a 325 pump system, a 430 double-wavelength UV–VIS detector set at 280 and 400 nm, and a 450-MT2 data system. The column was reversed-phase Lichrospher 100-RP18 (5 μm packing) (250 × 4 mm i.d.) protected with a guard column of same material (Merck). The UV–VIS spectra were recorded from 240 to 500 nm and the purity of each peak checked by means of a Millipore–Waters photodiode array detector (model 990) connected on-line to the HPLC system described above. Elution conditions were as follows: 1 ml min^{-1} flow rate; oven temp. 30°; solvent A, 2.5% aq. HOAc; solvent B, MeCN–solvent A (4:1); linear gradients from 5 to 20% B in 20 min and from 20 to 32% B in 12 min, followed by washing and reconditioning of the column.

NMR spectrometry. All NMR spectra were recorded in CD_3OD at 25° at 500.13 MHz for ^1H and 125.77 MHz for ^{13}C. Chemical shifts were reported in ppm relative to TMS.

ES-MS. Each fr., dissolved in MeOH–H_2O (1:1), was directly injected on to the MS system using a Harvard apparatus 11 (Model 55-1111) injection system with a flow rate of 0.3 ml hr^{-1}. All frs were analysed in the negative mode.

ES-MS and NMR data

Fraction I. ES-MS: *m/z* 599 $[(M-H)-H+Na]^-$ (13), 577 $[M-H]^-$ (100), 461 $[(M-H)-C_7H_6O_3-H+Na]^-$ (6), 447 $[(M-H)-C_8H_8O_3-H+Na]^-$ (12), 439 $[(M-H)-C_7H_6O_3]^-$ (17), 425 $[(M-H)-C_8H_8O_3]^-$ (9), 287 $[(M-H)-C_7H_6O_3-C_8H_8O_3]^-$ (7), 137 $[(C_7H_6O_3)-H]^-$ (15), 125 $[C_6H_6O_3]^-$ (13).

Fraction II (*Fig.* 1, **1** *or* **2**). ES-MS: *m/z* 599 $[(M-H)-H+Na]^-$ (6), 577 $[M-H]^-$ (100), 447 $[(M-H)-C_8H_8O_3-H+Na]^-$ (5), 439 $[(M-H)-C_7H_6O_3]^-$ (16), 425 $[(M-H)-C_8H_8O_3]^-$ (17), 289 $[(M-H)-C_{15}H_{12}O_6]^-$ (76), 137 $[(C_7H_6O_3)-H]^-$ (24), 125 $[C_6H_6O_3]^-$ (78). ^{13}C NMR: δ 28.2 (C – 4C), 28.6 (C – 4F), 68.7 (C – 3C), 68.7 (C – 3F), 82.7 (C – 2C), 82.7 (C – 2F), 95.5 (C – 6A or C – 8A), 96.4 (C – 6A or C – 8A), 96.6 (C – 6D), 100.8 (C – 4aA), 102.1 (C – 4aD), 115.3 (C – 2′B or C – 2′E), 115.1 (C – 2′B or C – 2′E), 116.1 (C – 5′E), 116.8 (C – 5′B), 120.1 (C – 6′E), 122.2 (C – 6′B), 125.4 (C – 8D), 131.8 (C – 1′E), 132.2 (C – 1′B), 145.9 (C – 3′E or C – 4′E), 146.1 (C – 3′E or C – 4′E), 147.2 (C – 3′B or C – 4′B), 148.2 (C – 3′B or C – 4′B), 149.2 (C – 8aD), 149.8 (C – 7D), 153.7 (C – 5D), 156.8 (C – 5A, C – 7A or C – 8aA), 157.5 (C – 5A, C – 7A or C – 8aA), 157.8 (C – 5A, C – 7A or C – 8aA). ^1H NMR; δ 2.39 (1H, *dd*, $J_{4\beta C-3C}$ = 7.9 Hz, $J_{4\beta C-4\alpha C}$ = 16.2 Hz, H – 4βC), 2.47 (1H, *dd*, $J_{4\beta F-3F}$ = 8.3 Hz, $J_{4\beta F-4\alpha F}$ = 16.2 Hz, H – 4βF), 2.70 (1H, *dd*, $J_{4\alpha C-3C}$ = 5.3 Hz, $J_{4\alpha C-4\beta C}$ = 16.2 Hz, H – 4αC), 2.78 (1H, *dd*, $J_{4\alpha F-3F}$ = 5.4 Hz, $J_{4\alpha F-4\beta F}$ = 16.2 Hz, H – 4αF), 3.84 (1H, *m*, H – 3C), 3.88 (1H, *m*, H – 3F), 4.44 (1H, *d*, J_{2F-3F} = 7.4 Hz, H – 2F), 4.48 (1H, *d*, J_{2C-3C} = 7.2 Hz, H – 2C), 5.71 (1H, *d*, J_{6A-8A} = 2.2 Hz, H – 6a or H – 8A), 5.83 (1H, *d*, J_{6A-8A} = 2.2 Hz, H – 6A or H – 8A), 5.98 (1H, *s*, H – 6D or H – 8D), 6.47 (1H, *dd*, $J_{6'E-5'E}$ = 8.2 Hz, $J_{6'E-2'E}$ = 2.0 Hz, H – 6′E), 6.57 (1H, *d*, $J_{5'E-6'E}$ = 8.2 Hz, H – 5′E), 6.62 (1H, *d*, $J_{2'E-6'E}$ = 2.0 Hz, H – 2′E), 6.71 (1H, *d*, $J_{5'B-6'B}$ = 8.1 Hz, H – 5′B), 6.74 (1H, *d*, $J_{2'B-6'B}$ = 1.8 Hz, H – 2′B), 6.76 (1H, *dd*, $J_{6'B-5'B}$ = 8.1 Hz, $J_{6'B-2'B}$ = 1.8 Hz, H – 6′B).

Fraction III. ES-MS: *m/z* 599 $[(M-H)-H+Na]^-$ (17), 577 $[M-H]^-$ (100), 461 $[(M-H)-C_7H_6O_3-H+Na]^-$ (7), 447 $[(M-H)-C_8H_8O_3-H+Na]^-$ (12), 439 $[(M-H)-C_7H_6O_3]^-$ (31), 425 $[(M-H)-C_8H_8O_3]^-$ (10), 287 $[(M-H)-C_7H_6O_3-C_8H_8O_3]^-$ (7), 137 $[(C_7H_6O_3)-H]^-$ (3), 125 $[C_6H_6O_3]^-$ (2).

Fraction IV. ES-MS: *m/z* 599 $[(M-H)-H+Na]^-$ (11), 577 $[M-H]^-$ (100), 461 $[(M-H)-C_7H_6O_3-H+Na]^-$ (5), 447 $[(M-H)-C_8H_8O_3-H+Na]^-$ (5), 439 $[(M-H)-C_7H_6O_3]^-$ (25), 425 $[(M-H)-C_8H_8O_3]^-$ (11), 287 $[(M-H)-C_7H_6O_3-C_8H_8O_3]^-$ (5), 137 $[(C_7H_6O_3)-H]^-$ (7), 125 $[C_6H_6O_3]^-$ (6).

Fraction V (*Fig.* 1, **1** *or* **2**). ES-MS: *m/z* 599 $[(M-H)-H+Na]^-$ (5), 577 $[M-H]^-$ (70), 447 [(M –

H) − $C_8H_8O_3$ − H + Na]$^-$ (5), 439 [(M − H) − $C_7H_6O_3$]$^-$ (3), 425 [(M − H) − $C_8H_8O_3$]$^-$ (7) 289 [(M − H) − $C_{15}H_{12}O_6$]$^-$ (100), (13), 137 [($C_7H_6O_3$) − H]$^-$ (49), 125 [$C_6H_6O_3$]$^-$ (79). ^{13}C NMR: δ 28.3 (C − 4F), 28.6 (C − 4C), 68.2 (C − 3F). 68.9 (C − 3C), 82.8 (C − 2C), 82.8 (C − 2F), 95.6 (C − 6A), 96.5 (C − 8A), 96.5 (C − 6D), 100.9 (C − 4aA), 102.0 (C − 4aD), 115.1 (C − 2′E), 116.0 (C − 2′B), 116.0 (C − 5′B), 116.0 (C − 5′E), 119.8 (C − 6′E), 119.8 (C − 6′B), 125.5 (C − 8D), 131.8 (C − 1′E), 134.7 (C − 1′B), 146.0 (C − 3′E or C − 4′E), 146.1 (C − 3′E or C − 4′E), 147.3 (C − 3′B or C − 4′B), 148.2 (C-3′B or C − 4′B), 149.1 (C − 8aD), 150.0 (C − 7D), 153.7 (C − 5D), 156.9 (C − 8aA), 157.6 (C − 5A), 157.9 (C − 7A). ^{1}H NMR: δ 2.51 (1H, *dd*, $J_{4\beta C-3C}$ = 8.4 Hz, $J_{4\beta C-4\alpha C}$ = 16.1 Hz, H − βC), 2.59 (1H, *dd*, $J_{4\beta F-3F}$ = 7.8 Hz, $J_{4\beta F-4\alpha F}$ = 16.2 Hz, H − 4βF), 2.85 (1H, *dd*, $J_{4\alpha C-3C}$ = 5.5 Hz, $J_{4\alpha C-4\beta C}$ = 16.1 Hz, H − 4αC), 2.87 (1H, *dd*, $J_{4\alpha F-3F}$ = 5.3 Hz, $J_{4\alpha F-4\beta F}$ = 16.2 Hz, H − 4αF), 3.96 (1H, *m*, H − 3F), 3.97 (1H, *m*, H − 3C), 4.59 (1H, *d*, J_{2C-3C} = 7.6 Hz, H − 2C), 4.61 (1H, *d*, J_{2F-3F} = 7.1 Hz, H − 2F), 5.87 (1H, *d*, J_{6A-8A} = 2.3 Hz, H − 6A), 5.93 (1H, *d*, J_{8A-6A} = 2.3 Hz, H − 8A), 6.09 (1H, *s*, H − 6D), 6.56 (1H, *dd*, $J_{6'E-5'E}$ = 8.2 Hz, $J_{6'E-2'E}$ = 2.0 Hz, H-6′E), 6.68 (1H, *d*, $J_{2'E-6'E}$ = 1.9 Hz, H − 2′E), 6.70 (1H, *d*, $J_{5'E-6'E}$ = 8.2 Hz, H − 5′E), 6.73 (1H, *d*, $J_{5'B-6'B}$ = 8.3 Hz, H − 5′B), 6.75 (1H, *dd*, $J_{6'B-5'B}$ = 8.3 Hz, $J_{6'B-2'B}$ = 1.8 Hz, H-6′B), 6.89 (1H, *d*, $J_{2'B-6'B}$ = 1.8 Hz, H − 2′B).

Fraction VI (*Fig.* 1, **3**). ES-MS: *m/z* 599 [(M − H) − H + Na]$^-$ (30), 577 [M − H]$^-$ (100), 461 [(M − H) − $C_7H_6O_3$ − H + Na]$^-$ (10), 447 [(M − H) − $C_8H_8O_3$ − H + Na]$^-$ (14), 439 [(M − H) − $C_7H_6O_3$]$^-$ (21), 425 [(M − H) − $C_8H_8O_3$]$^-$ (8), 289 [(M − H) − $C_{15}H_{12}O_6$]$^-$ (7), 287 [(M − H) − $C_7H_6O_3$ − $C_8H_8O_3$]$^-$ (5), 137 [($C_7H_6O_3$) − H]$^-$ (26), 125 [$C_6H_6O_3$]$^-$ (10). ^{13}C NMR: δ 26.3 (C − 4C), 29.1 (C − 4F), 67.5 (C − 3C), 68.6 (C − 3F), 80.4 (C − 2C), 83.1 (C − 2F), 95.6 (C − 6A or C − 8A), 96.2 (C − 6D or C − 8D), 96.2 (C − 6A or C − 8A), 100.5 (C − 4aA), 101.5 (C − 4aD), 108.4 (C − 8D or C − 6D), 114.1 (C − 2′B), 116.1 (C − 2′E), 116.2 (C − 5′E), 120.0 (C − 6′E), 120.4 (C − 5′B), 126.5 (C − 6′B), 131.7 (C − 1′B), 132.1 (C − 1′E), 145.7 (C − 3′B), 145.7 (C − 4′B), 145.9 (C − 3′E or C − 4′E), 146.1 (C − 3′E or C − 4′E), 154.6 (C − 7D), 155.8 (C − 8aD or C − 5D), 157.0 (C − 8aD or C − 5D), 157.7 (C − 8aA), 158.0 (C − 5A), 158.0 (C − 7A). ^{1}H NMR: δ 2.47 (1H, *dd*, $J_{4\alpha C-3C}$ = 6.1 Hz, $J_{4\alpha C-4\beta C}$ = 16.2 Hz, H − 4αC), 2.59 (1H, *dd*, $J_{4\beta F-3F}$ = 13.2 Hz, $J_{4\beta F-4\alpha F}$ = 16.2 Hz, H − 4βF), 2.60 (1H, *dd*, $J_{4\beta C-3C}$ = 9.7 Hz, $J_{4\beta C-4\alpha C}$ = 16.2 Hz, H − 4βC), 2.93 (1H, *dd*, $J_{4\alpha F-3F}$ = 5.6 Hz, $J_{4\alpha F-\beta F}$ = 16.2 Hz, H − 4αF), 3.99 (1H, *m*, H − 3F), 4.10 (1H, *m*, H − 3C), 4.53 (1H, *d*, J_{2F-3F} = 7.5 Hz, H − 2F), 4.85 (1H, *d*, J_{2C-3C} = 5.5 Hz, H − 2C), 5.88 (1H, *d*, J_{6A-8A} = 2.4 Hz, H − 6A or H − 8A), 5.90 (1H, *d*, J_{6A-8A} = 2.4 Hz, H − 8A or H − 6A), 6.06 (1H, *s*, H − 6D or H − 8D), 6.55 (1H, *s*, H − 5′B), 6.63 (1H, *dd*, $J_{6'E-5'E}$ = 8.2 Hz, $J_{6'E-2'E}$ = 2.1 Hz, H − 6′E), 6.70 (1H, *d*, $J_{5'E-6'E}$ = 8.2 Hz, H − 5′E), 6.74 (1H, *d*, $J_{2'E-6'E}$ = 2.1 Hz, H − 2′E), 6.82 (1H, *s*, H − 2′B).

Fraction VII (*Fig.* 1, **5**). ES-MS: *m/z* 597 [(M − H) − H + Na]$^-$ (8), 575 [M − H]$^-$ (100), 445 [(M − H) − $C_8H_8O_8$ − H + Na]$^-$ (25), 437 [(M − H) − $C_7H_6O_3$]$^-$ (12), 423 [(M − H) − $C_8C_8H_8O_3$]$^-$ (15), 394 [(M − H) − $C_9H_9O_4$]$^-$ (39), 271 [(M − H) − $C_7H_6O_3$ − $C_8H_7O_3$ − CH_2]$^-$ (29), 243 [(M − H) − $C_9H_9O_4$ − $C_8H_7O_3$]$^-$ (13), 229 [(M − H) − $C_9H_9O_4$ − $C_8H_7O_3$ − CH_2]$^-$ (13), 137 [($C_7H_6O_3$) − H]$^-$ (33), 125 [$C_6H_6O_3$]$^-$ (24). ^{13}C NMR: δ 26.6 (C − 4F), 27.8 (C − 4C), 67.2 (C − 3C), 68.0 (C − 3F), 79.6 (C − 2C), 83.6 (C − 2F), 91.9 (C − 6D or C − 8D), 92.1 (C − 1′B), 95.4 (C − 6A or C − 8A), 97.4 (C − 6A or C − 8A), 100.0 (C − 4aA), 104.8 (C − 4aD), 105.0 (C − 8D or C − 6D), 115.1 (C − 2′E), 115.1 (C − 5′B), 116.5 (C − 5′E), 119.8 (C − 6′E), 131.3 (C − 1′E), 146.6 (C − 3′E), 146.6 (C − 4′E), 154.6 (C − 8aA), 155.5 (C − 8aD), 157.1 (C − 5A or C − 7A)), 157.8 (C − 5A or C − 7A), 164.8 (C − 6′B), 167.6 (C − 7D), 180.8 (C − 4′B), 191.0 (C − 3′B), invisible (C − 2′B), invisible (C − 5D). ^{1}H NMR: δ 2.45 (1H, *dd*, $J_{4\beta C-3C}$ = 7.3 Hz, $J_{4\beta C-4\alpha C}$ = 16.2 Hz, H − 4βC), 2.58 (1H, *dd*, $J_{4\beta F-3F}$ = 7.8 Hz, $J_{4\beta F-4\alpha F}$ = 16.3 Hz, H − 4βF), 2.69 (1H, *dd*, $J_{4\alpha C-3C}$ = 5.2 Hz, $J_{4\alpha C-4\beta C}$ = 16.2 Hz, H − 4αC), 2.85 (1H, *dd*, $J_{4\alpha F-3F}$ = 5.2 Hz, $J_{4\alpha F-4\beta F}$ = 16.3 Hz, H − 4αF), 3.10 (1H, *d*, $J_{2'\alpha B-2'\beta B}$ = 15.6 Hz, H − 2′B), 3.64 (1H, *d*, $J_{2'\beta B-2'\alpha B}$ = 15.6 Hz, H − 2′B), 3.79 (1H, *m*, H-3C), 4.08 (1H, *m*, H − 3F), 4.29 (1H, *d*, J_{2C-3C} = 6.3 Hz, H − 2C), 4.86 (1H, *d*, J_{2F-3F} = 7.1 Hz, H − 2F), 5.54 (1H, *d*, J_{8A-6A} = 2.3 Hz, H − 8A or H − 6A), 5.88 (1H, *d*, J_{6A-8A} = 2.3 Hz, H − 6A or H − 8A), 6.08 (1H, *s*, H − 6D or H − 8D), 6.61 (1H, *s*, H − 5′B), 6.76 (1H, *dd*, $J_{6'E-5'E}$ = 8.2 Hz, $J_{6'E-2'E}$ = 2.0 Hz, H − 6′E), 6.81 (1H, *d*, $J_{5'E-6'E}$ = 8.2 Hz, H − 5′E), 6.87 (1H, *d*, $J_{2'E-6'E}$ = 2.0 Hz, H − 2′E).

Fraction VIII (*Fig.* 1, **8**). ES-MS: *m/z* 575 [M − H]$^-$ (100), 445 [(M − H) − $C_8H_8O_3$ − H + Na]$^-$ (10), 423 [(M − H) − $C_8H_8O_3$]$^-$ (12), 394 [(M − H) − $C_9H_9O_4$]$^-$ (26), 271 [(M − H) − $C_7H_6O_3$ − $C_8H_7O_3$ − CH_2]$^-$ (30), 243 [(M − H) − $C_9H_9O_4$ − $C_8H_7O_3$]$^-$ (23), 229 [(M − H) − $C_9H_9O_4$ − $C_8H_7O_3$ − CH_2]$^-$ (11), 137 [($C_7H_6O_3$) − H]$^-$ (11), 125 [$C_6H_6O_3$]$^-$ (19). ^{13}C NMR: δ 27.8 (C − 4C), 28.3 (C − 4F), 46.0 (C − 2′B), 67.2 (C − 3C), 67.9 (C − 3F), 79.6 (C − 2C), 83.5 (C2F), 89.9 (C1′B), 91.0 (C − 6D), 95.3 (C − 3′B), 95.9 (C − 6A or C − 8A), 97.1 (C − 6A or C − 8A), 100.5 (C − 4aA), 104.0 (C − 4aD), 106.0 (C − 8D), 112.8 (C − 5′B), 114.9 (C − 2′E), 116.4 (C − 5′E), 119.7 (C − 6′E), 131.3 (C − 1′E), 145.6 (C − 3′E or C − 4′E), 146.5 (C − 3′E or C-4′E), 155.1 (C − 8aD), 156.6 (C − 8aA or C − 5A or C − 7A), 158.0 (C − 8aA, C − 5A or C − 7A), 158.6 (C − 8aA, C − 5A or C − 7A), 164.3 (C − 6′B), 166.3 (C − 5D), 168.1 (C − 7D), 194.3 (C − 4′B). ^{1}H NMR: δ 2.49 (1H, *d*, $J_{2'\beta B-2'\alpha B}$ = 11.7 Hz, H-2′B), 2.52 (1H, *dd*, $J_{4\beta C-3C}$ = 8.3 Hz, $J_{4\beta C-4\alpha C}$ = 15.2 Hz, H − 4βC), 2.60 (1H, *dd*, $J_{4\beta F-3F}$ = 7.5 Hz, $J_{4\beta F-4\alpha F}$ = 16.3 Hz,

H − 4βF), 2.68 (1H, *d*, $J_{2'\alpha B-2'\beta B}$ = 11.7 Hz, H − 2'B), 2.86 (1H, *dd*, $J_{4\alpha F-3F}$ = 5.2 Hz, $J_{4\alpha F-4\beta F}$ = 16.3 Hz, H − 4αF), 2.94 (1H, *dd*, $J_{4\alpha C-3C}$ = 5.6 Hz, $J_{4\alpha C-4\beta C}$ = 15.2 Hz, H − 4αC), 3.98 (1H, *m*, H − 3c), 3.98 (1H, *d*, J_{2C-3C} = 7.2 Hz, H − 2C), 4.11 (1H, *m*, H − 3F), 4.93 (1H, *d*, J_{2F-3F} = 7.0 Hz, H − 2F), 5.55 (1H, *d*, J_{6A-8A} = 2.4 Hz, H − 6A or H − 8A), 5.90 (1H, *d*, J_{8A-6A} = 2.4 Hz, H − 8A or H − 6A), 6.12 (1H, *s*, H − 6D), 6.43 (1H, *s*, H − 5'B), 6.74 (1H, *dd*, $J_{6'E-5'E}$ = 8.1 Hz, $J_{6'E-2'E}$ = 2.0 Hz, H − 6'E), 6.79 (1H, *d*, $J_{5'E-6'E}$ = 8.1 Hz, H − 5'E), 6.85 (1H, *d*, $J_{2'E-6'E}$ = 2.0 Hz, H − 2'E).

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