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STRUCTURE OF OLIGOMERIC PROANTHOCYANIDINES FROM *Hedysarum thienum* ROOTS STUDIED BY THIOLYSIS AND MALDI-TOF MS

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Oligomeric proanthocyanidines from Hedysarum theinum *roots that were cleaved by benzylmercaptan were shown to be heterogeneous mixtures consisting of prodelphinine and procyanidine structural units with the former dominating. Fractionation of oligomeric proanthocyanidines on polyamide and MCI gel CHP20P sorbent isolated fractions containing primarily mixtures of di- and trimeric, tri- and tetrameric, or tetra- and pentameric proanthocyanidines. Analysis by mass spectrometry (MALDI-TOF) showed that fractions of trimers and tetramers contained more proanthocyanidines with a single A-type bond than fractions of dimers and trimers.*

Key words: *Hedysarum theinum* Krasnob., Fabaceae, oligomeric proanthocyanidines, A-type proanthocyanidines, condensed tannins, acid cleavage, thiolysis, mass spectrometry, MALDI-TOF.

In continuation of research on the chemical composition of the rare and endangered plant *Hedysarum theinum* Krasnob. [1], which possesses valuable medicinal properties, we turned our attention to the fact that a significant part of the substances extracted from roots of this plant are oligomeric proanthocyanidines (oPA, condensed tannins) (Fig. 1). Thus, it was noted previously that the yield of extracted substances was about 15-20% after exhaustive methanol extraction of the roots [2]. Spectrophotometric determination of oPA in the methanol extract by reaction with vanillin/HCl gave an oPA content at the 90-95% level [2]. Despite this inflated value, the results indicate that oPA are one of the main extractable substances from roots of *H. theinum*.

A literature search shows that rather homogeneous oPA could be separated preparatively into pure components of di- and trimeric [3] and sometimes tetra- and pentameric proanthocyanidines [4]. Heterogeneous oPA typically have a medium-length oPA chain and a ratio of structural units that can be established, for example, by depolymerization under acidic conditions in the presence of nucleophiles such as benzylmercaptan [5, 6], thiophenol [4], cysteamine [7], or phloroglucinol [8]. The C-8—C-4 bond is cleaved during the reaction at a random position of the oPA. The nucleophile substitutes at the C-4 position of the resulting "upper" fragment. Only the terminal unit is obtained in an unsubstituted form upon full cleavage of the oPA. The middle units give 4-substituted derivatives. The ratio of substituted and unsubstituted products enables the average degree of oPA polymerization to be found. Furthermore, an advantage of this approach is the preservation of information about the stereochemistry of the C-2 and C-3 centers of the oPA units. The analytical method for oPA has been described in detail [9, 10].

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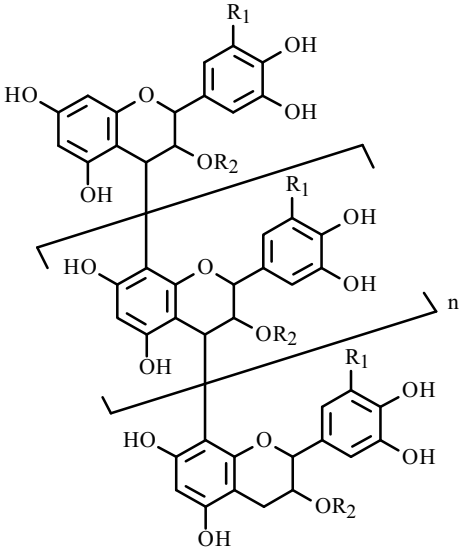


R_1 = H, OH; R_2 = H, -CO-2,4,6-$(OH)_3$-C_6H_2

Fig. 1. General structure of B-type oPA.

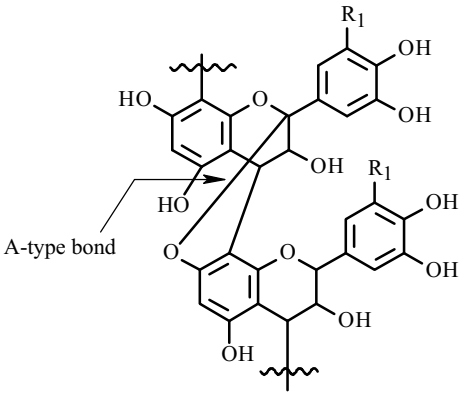


Fig. 2. Fragment of oPA containing an A-type bond.

Direct determination of the molecular weight by MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight) mass spectrometry [11] and ESI (electrospray ionization) [12] have relatively recently been used to establish the structure of oPA. These do not give fragmentation products and enable the molecular weight of each compound in the oPA mixture to be found. This, in combination with data from the nucleophilic depolymerization, helps to establish its structure. The MALDI-TOF and ESI-MS methods can easily determine the presence of A-type bonds in the oPA (Fig. 2). The molecular weight of polymer with A-type bonds will be (2 × m) Da less than a B-type oPA, where m is the number of A-type bonds in the molecule.

We studied oPA extracted by ethanol from *H. theinum* roots. The dried alcohol extract was extracted in a Soxhlet apparatus successively with $CHCl_3$ and EtOAc to remove low-molecular-weight compounds. The separation procedure has been described in detail [1]. Extraction of the remainder in a Soxhlet apparatus with acetone produced an acetone fraction. The remainder after extraction was dissolved in ethanol to produce an alcohol fraction of the ethanol extract of *H. theinum* roots. The resulting fractions give a dark red color upon reaction with vanillin in HCl (test for oPA).

The acetone fraction was preliminarily separated over polyamide to produce fractions 3 and 4 with oPA (see Experimental). Polyaromatic resin MCI gel CHP20P, which has been used for analogous purposes [13-15] was used to separate the oPA. Column chromatography over MCI gel CHP20P that was intended to separate fractions 3 and 4 produced several narrower fractions containing a mixture of oPA according to HPLC. The order of elution of oPA from the MCI gel CHP20P column correlated with the order of elution on reversed-phase (RP) ProntoSilC18 by HPLC.

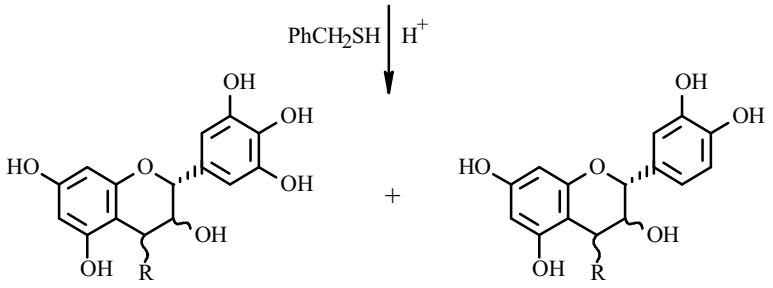
The resulting narrow fractions of OPA were reacted with benzylmercaptan and HCl under the literature conditions [12]. This gave rapid and complete depolymerization of oPA. The composition of the reaction mixtures was analyzed by HPLC without processing. The qualitative composition of the reaction mixtures obtained after thiolysis of the various oPA fractions was identical. The thiolysis products decomposed in the presence of HCl during processing of the reaction mixture and separation. Therefore, depolymerization products were identified using a scaling thiolysis reaction of oPA with benzylmercaptan and acetic acid under conditions analogous to those in the literature [5]. Separation by preparative HPLC over RP DiasorbC16 isolated the pure compounds gallocatechin (**1**), epigallocatechin (**2**), catechin (**3**), epicatechin (**4**), (3,4-*trans*)-4-benzylthiogallocatechin (**5**) (in a mixture with **4** at a 1.1:1 ratio), (3,4-*cis*)-4-benzylthiogallocatechin (**6**), (3,4-*trans*)-4-benzylthioepigallocatechin (**7**), (3,4-*cis*)-4-benzylthiocatechin (**8**), and (3,4-*trans*)-4-benzylthioepicatechin (**9**) (Scheme 1). Neither 3-*O*-gallates of flavanols nor their thio-derivatives or gallic acid were found among the reaction products. According to the literature, loss of gallic acid from 3-*O*-gallates should not occur under these reaction conditions [15, 16].

TABLE 1. ^{13}C NMR Spectra of **5-9** (75 MHz, acetone-d_6, δ, ppm)

C atom	5	6	7	8	9
1a	157.50	155.10	156.25	155.60	156.33
2	75.60	77.77	74.58	78.11	74.56
3	71.32	70.87	70.35	71.21	70.30
4	43.60	43.02	42.38	43.75	42.60
4a	100.61	100.56	99.02	101.14	99.00
5	158.42*	155.99	157.59	156.52	157.64
6	97.32	95.22	95.86	95.74	95.91
7	158.50*	157.98	158.09	158.50	158.14
8	94.51	94.03	94.82	94.53	94.87
1′	129.67	128.69	130.37	130.58	131.19
2′	106.73	106.87	106.03	115.23*	114.51*
3′	145.49	144.65	145.42	145.22**	144.57**
4′	132.78	132.35	132.16	144.85**	144.70**
5′	145.49	144.95	145.42	114.82*	114.71*
6′	106.73	106.87	106.03	120.15	118.42
S**C**H$_2$	34.89	37.74	36.30	38.26	36.37
1″	139.37	138.84	139.04	139.34	139.19
2″,6″	129.21	128.69	128.99	129.20	129.00
3″,5″	128.46	127.78	128.42	128.30	128.44
4″	126.96	126.19	126.85	126.71	126.84

Assignments of resonances marked * and ** may be interchanged within a single column.

Oligomeric proanthocyanidines



1, 2, 5 - 7 **3, 4, 8, 9**

1, 3, 5, 6, 8: 3–*β*–OH; **2, 4, 7, 9:** 3–*α*–OH

1 - 4: R = H; **5:** R = *α*–SCH$_2$Ph; **6 - 9:** R = *β*–SCH$_2$Ph

Scheme 1

Compounds **1**-**4** were identified using PMR spectra and comparison with literature data [1, 17]. Compounds **3** and **4** were characterized by HPLC using spectral data and retention times for authentic samples. Compounds **5**-**9** were identified based on a comparison of PMR and ^{13}C NMR data with the literature values [13, 14]. The SSCC for protons of the (3,4-*trans*)-thio-derivative of gallocatechin (**5**) were $J_{H\text{-}2,H\text{-}3}$ = 7 Hz and $J_{H\text{-}4,H\text{-}3}$ = 6 Hz [13]. The SSCC differed for (3,4-*cis*)-thio-derivatives of gallocatechin (**6**) and catechin (**8**) and were $J_{H\text{-}2,H\text{-}3}$ = 10 Hz and $J_{H\text{-}4,H\text{-}3}$ = 4-5 Hz [13, 14]. The SSCC was $J_{H\text{-}4,H\text{-}3}$ = 2 Hz and $J_{H\text{-}2,H\text{-}3}$ was not determined for (3,4-trans)-thio-derivatives of epigallocatechin (**7**) and epicatechin (**9**) [14]. We present the ^{13}C NMR spectra of **5**-**8** for the first time (Table 1). We recorded ^{1}H NMR double resonance and 2D ^{13}C—^{1}H NMR spectra for **6**. Unfortunately, the measured rotation angles $[\Delta]_{580}$ for **5**-**9** were of the order of the instrument measurement uncertainty (±10) so that a definite conclusion about their configurations could not be made. We previously isolated from *H. theinum* roots (-)-catechin (**3**) ($[\Delta]_{580}$ -4.76θ) and (-)-epicatechin (**4**) ($[\Delta]_{580}$ -27.75θ) [1].

TABLE 2. Composition of oPA Fractions, % from Thiolysis Results

Fraction No.	Terminal unit			Middle units					Average degree of polymerization	pD/pC
	1	2+3	4	5	6	7	8	9		
3-2-2	15.0	17.0	0.7	4.0	17.2	12.5	2.1	1.3	2.1	9.9
3-2-3	4.2	28.5	4.3	3.0	9.5	4.1	1.0	0.9	1.5	8.7
3-2-4	6.9	21.4	10.5	4.3	18.6	5.4	6.5	2.3	2.0	3.2
3-2-5	4.9	15.7	11.2	3.6	13.6	6.4	7.6	2.8	2.1	2.3
3-2-6	4.9	23.1	11.1	3.1	13.8	7.6	8.0	4.7	2.0	1.9
3-2-7	2.1	12.1	6.4	2.8	8.3	4.1	5.5	3.6	2.2	1.7
3-2-8	2.7	12.3	9.3	3.7	11.9	5.5	6.2	5.7	2.4	1.8
3-2-9	0.4	3.6	6.0	7.8	8.2	3.2	5.9	3.2	3.8	2.1
3-4	2.2	18.6	11.8	3.5	11.8	4.9	6.1	2.3	1.9	2.4
3-5	1.0	8.0	10.7	3.5	7.7	3.7	6.4	4.7	2.3	1.3
4-1-3	9.6	7.9	0.7	2.1	11.4	10.2	1.8	1.2	2.5	7.9
4-1-4	9.7	16.0	2.5	4.4	21.0	10.6	2.7	2.0	2.4	7.7
4-1-5	8.0	15.1	3.1	4.6	24.3	12.1	4.9	3.1	2.9	5.1
4-1-6	7.1	3.1	4.8	5.8	23.1	14.5	7.5	4.4	4.7	3.6
4-1-7	3.2	10.8	5.2	5.1	18.0	12.4	8.2	5.0	3.5	2.7
4-1-8	3.3	11.6	6.4	6.1	16.1	10.7	7.9	6.0	3.2	2.4
4-2	9.5	15.2	5.3	4.0	18.6	11.2	5.4	3.4	2.4	3.8
4-3	6.5	16.9	7.0	3.7	18.5	11.7	7.0	4.5	2.5	2.9
4-4	2.5	11.8	9.0	3.9	20.6	14.1	11.1	8.4	3.5	2.0
sp2	1.3	4.8	4.2	6.0	14.0	7.3	7.0	3.4	4.7	2.6
sp3	3.9	9.0	4.7	5.8	20.0	14.6	7.4	4.5	4.0	3.4

According to the literature, thiolysis of the structural units in **1** and **3** in the oPA give two epimers of the thio-derivative (3,4-*cis*- and 3,4-*trans*-) [3, 5]. Only the 3,4-*trans*-isomer of the thio-derivative **7** has been described for epigallocatechin (**2**) [14]. There is disagreement regarding thio-derivatives of epicatechin (**4**). In most studies only the 3,4-*trans*-isomer **9** was observed [3, 5, 13, 14]. However, the 3,4-*cis*-isomer of 4-benzylthioepicatechin has also been mentioned [18]. We found two isomers of 4-benzylthiogallocatechin, **5** and **6**, and one isomer of 4-benzylthioflavanols **7**-**9**. The hypothetical possibility that minor amounts of the other isomers of the thioethers were present in the mixture would not cause significant changes in the calculated average degree of polymerization of the fractions.

As mentioned above, the qualitative composition of the products obtained after thiolysis of different oPA fractions was the same. Table 2 gives the quantitative data. The relative content for a model mixture of **3** + **4** + **8** agreed with the PMR and HPLC. This allowed quantitative data obtained by HPLC to be used to determine the mole ratios of the compounds. HPLC under these conditions cannot always clearly separate peaks for **2** and **3**. Therefore, we give their total value. However, this did not interfere with the calculation of the average degree of oPA polymerization, equal to 1 + the ratio of the sum of middle units to the sum of terminal ones. Another important characteristic of oPA is the ratio of prodelphinine (pD) units incorporating derivatives of gallocatechin (**1**) and epigallocatechin (**2**) and procyanidine (pC) units incorporating derivatives of catechin (**3**) and epicatechin (**4**), which was calculated from the ratio (**5** + **6** + **7**)/(**8** + **9**).

Table 2 suggests that separation over MCI gel CHP20P sorbent occurs in the order of decreasing content of pD units in oPA. Fractions 3-2-2 and 3-2-3, for which the ratio pD/pC was 10/9, were eluted first. Then, fractions 3-2-4 through 3-2-8 and 3-4 and 3-5, for which the ratio pD/pC was in the range from 3.2 to 1.3, were eluted. In analogy, pD/pC was 7.9-7.7 for fractions 4-1-3 through 4-1-4 and 5.1-2.0 for fractions 4-1-5 through 4-4. The average degree of polymerization for fractions 3-2-2, 3-2-4 through 3-2-8, 3-4, and 3-5 was in the range 1.9-2.4; for fractions 4-1-3 through 4-4, in the broader range 2.4-4.7. HPLC traces of fractions eluted by 75 and 100% methanol had additional peaks that interfered with the determination of the thiolysis products. This may have been due to the presence in them of compounds other than oPA.

The alcohol fraction of the ethanol extract was separated analogously over polyamide. Fractions 2 and 3 of the alcohol fraction underwent thiolysis without additional separation because the analytical results for fractions 3 and 4 of the acetone fraction showed only partial separation of oPA. The average degree of polymerization of fraction sp2 was greater than that of fraction sp3, 4.7 and 4.0, respectively (Table 2).

TABLE 3. Composition of oPA Fractions from MALDI-TOF Mass Spectra

Degree of polymerization	oPA composition	$[M + Na]^+$ calc.	$[M + Na]^+$ expt.	Fraction No.						
				3-2-2	3-2-7	3-4	4-1-3	4-1-5	4-1-7	4-4
2	pC+pD	617.13	617.35	+++			+++			
2	2pD	633.12	633.35	+++			+++			
3	3pC	889.2	888.97		+++					++
3	2pC+pD-A	903.17	903.37		++					++
3	2pC+pD	905.19	905.80	+	+++	+++		++	+++	+++
3	pC+2pD-A	919.17	919.35		++	++			++	++
3	pC+2pD	921.19	921.70	++	+++	+++	++	+++	+++	+++
3	3pD-A	935.16	935.37	+	++	++	++		+	++
3	3pD	937.18	937.66	++		+++	+++	+++	+	++
4	3pC+pD-A	1191.24	1191.30							++
4	3pC+pD	1193.25	1192.78		++	++			+++	+++
4	2pC+2pD-A	1207.23	1207.30		++	++				++
4	2pC+2pD	1209.25	1209.83	+	++	++	+	+++	+++	+++
4	pC+3pD-A	1223.23	1223.89	+	++	++	++			++
4	pC+3pD	1225.24	1225.55	+	++	++	++	+++	+++	++
4	4pD-A	1239.22	1239.80	+		++	++			+
4	4pD	1241.24	1241.35	+		++	++	+++	++	+
5	4pC+pD	1481.32	1480.82		+				++	+
5	3pC+2pD-A	1495.30	1496.02		+	+				+
5	3pC+2pD	1497.31	1497.31			+			+++	++
5	2pC+3pD-A	1511.29	1511.26		+	+			++	+
5	2pC+3pD	1513.31	1513.31	+		+		++	+++	++
5	pC+4pD-A	1527.29	1527.52		+	+	+			+
5	pC+4pD	1529.30	1529.37	+		+	+	++	++	+
5	5pD-A	1543.28	1543.15			+	+			
5	5pD	1545.30	1545.12	+		+	+	+		
6	4pC+2pD-A	1783.36	1783.11							+
6	4pC+2pD	1785.38	1785.26						+	+
6	3pC+3pD-A	1799.35	1799.08							+
6	3pC+3pD	1801.37	1801.25					+	+	+
6	2pC+4pD-A	1815.35	1815.09							+
6	2pC+4pD	1817.37	1817.23					+	+	+
6	pC+5pD	1833.36	1833.25					+	+	
6	6pD	1849.36	1849.23					+		

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pC, procyanidine unit; pD, prodelphinine unit; A, A-type bond present; +++, strong peak; ++, medium peak; +, weak peak.

Mass spectral data (MALDI-TOF) (Table 3) gave more accurate values of molecular weights and content of various oligomers in certain studied fractions. The resulting mass spectra contained groups of peaks corresponding to $[M + Na]^+$ adducts of compounds differing by 16 Da. Groups differed from each other by 288 Da. This was the characteristic weight of a procyanidine unit in a B-type oPA. The difference of molecular weight by 16 Da may have been due to 1) substitution of a procyanidine unit by prodelphinine (addition of an O atom to the molecule, +16); 2) substitution of three procyanidine units ($3 \times 288 = 864$ Da) by two galloprocyanidine units [$2 \times (288 + 152) = 880$], however, this possibility was unsatisfactory because thiolytic cleavage did not show differences of flavanol-3-*O*-gallates in oPA molecules; 3) the difference of 16 Da may have been due to substitution of $[M + Na]^+$ by $[M + K]^+$, 39 - 23 = 16 Da, however, this would not explain the presence in the mass spectra of clusters of peaks [M + Na + 16 × n], n = 2 and 3. It would be expected for preferential substitution of $[M + Na]^+$ by $[M + K]^+$ that the relative intensity of peaks differing by one structural unit (288) would be proportional. However, in our instance the intensities of similar peaks changed, which enabled this hypothesis to be discarded.

Peaks differing by 2 Da were observed in mass spectra of some fractions, for example, 919.35 and 921.70 (fractions 3-4, 4-1-7, 4-4); 935.37 and 937.66 (fractions 3-4, 4-1-3, 4-4), etc. The peak with higher mass was stronger. Such a difference

of masses corresponds often to the isotopic distribution of masses for molecules that contain chlorine. However, elemental analysis of the studied fractions did not find halogens. This led to the conclusion that the examined pairs of peaks belonged to two different compounds, for example $[C_{45}H_{36}O_{21} + Na]^+$ for M = 935.37 and $[C_{45}H_{38}O_{21} + Na]^+$ for M = 937.66. This corresponded to proanthocyanidine molecules with one A-type bond and without such a bond.

Therefore, MALDI-TOF mass spectra indicated that oPA from *H. theinum* were constructed exclusively from prodelphinine and procyanidine structural units. The studied fractions contained oPA with degree of polymerization from 2 to 6. The main compounds in fractions 3-2-2 through 3-4 were dimeric and trimeric PA whereas those in fractions 4-1-3 through 4-4 were tri-, tetra-, and pentamers. Table 3 shows that fractions 3-2-2 and 4-1-3 had a high content of prodelphinine structural units due to oligomers of composition pC + pD, 2pD, pC + 2pD, 3pD, 4pD, and 5pD. This agreed with results of thiolytic cleavage. The content of prodelphinine structural units in the last fractions decreased owing to an increase in the content of trimers 2pC + pD and tetramers 3pC + pD and 2pC + 2pD although the prodelphinine units still dominated. These results also correlated with thiolysis data. Fractions 4-1-3 through 4-4 had a larger content of oPA with one A-type bond than fractions 3-2-2 through 3-4. Mass spectra of fractions eluted from the column of MCI gel CHP20P by 75 and 100% methanol exhibited peaks in mass range 570-1000 that did not correspond to oPA masses.

Thus, we obtained for the first time structural data for oPA from *H. theinum* roots by using thiolytic cleavage and MALDI-TOF mass spectrometry. oPA from the roots are constructed of prodelphinine and procyanidine units. 3-*O*-Gallates of flavanols were not found. The prodelphinine units in these oPA dominate over procyanidine. This is rather rare [19]. The main compounds were dimers and trimers of PA in fraction 3 of the acetone fraction; trimers and tetramers of PA in fraction 4 of the acetone fraction; tetra- and pentamers of PA in the alcohol fraction. These results indicate that oPA from *H. theinum* roots differ significantly in composition from the well studied oPA from grape, for which the molecules are constructed from procyanidine units and their 3-*O*-gallates [3, 11, 16].

According to the literature, oPA exhibit a broad spectrum of biological activity including antimicrobial and immunomodulating [20, 21] and antiviral and anti-HIV [22]. Furthermore, oPA decrease pressure and sugar in blood [23] and lower the cholesterol level [24]. It can be assumed that the biological activity of *H. theinum* preparations is due to the presence of oPA as the main component and a complex of minor isoflavonoids.

EXPERIMENTAL

General Comments. PMR and ^{13}C NMR spectra of acetone-d_6 solutions (5-10%) were recorded on Bruker AV-300 (300.13 MHz for ^1H; 75.47, ^{13}C) and DRX-500 (500.13 MHz for ^1H; 125.76, ^{13}C) instruments. Optical rotation angles were measured on a Polamat A instrument. Mass spectra were recorded in a Reflex III MALDI-TOF mass spectrometer (Bruker, Germany) in reflection mode with generation of positively charged ions. A VSL-337 ND nitrogen laser (Laser Science Inc., USA) was used to ionize the molecules. The matrix was a saturated solution of 2,5-dihydroxybenzoic acid in CH_3CN. The external standard masses were $[M + H]^+$ ions of a bradykinin fragment (1-7), human angiotensin II, synthetic peptide P14R, a human adrenocorticotropic hormone fragment (18-39), and the B-chain of oxidized bovine insulin.

HPLC was carried out in a Milikhron A-02 microcolumn chromatograph (ZAO EkoNova, Novosibirsk) using a standard chromatographic column (2 × 75 mm) packed with ProntoSIL 120-5-C18 reversed-phase sorbent (5 μm, Bischoff, Germany) and gradient elution with simultaneous multi-wavelength detection at six wavelengths (220, 240, 260, 280, 320, and 360 nm) [25]. The eluent was A (methanol) and B (0.1% CF_3COOH in H_2O). Method 1 used a gradient of A: 0-30%, 5 min; 30-50%, 5 min; 50-70, 70% for 10 min; 70-90, 90% for 10 min; to 100% for 5 min. Method 2 used a gradient of A: 0-70%, 16 min. The temperature was 35°C; pressure, 30-36 atm; flow rate 150 μL/min. UV spectra were recorded on a Milikhrom A-02 instrument.

Column chromatography was performed over polyamide TU 6-09-10-822-73, particle size 0.25-0.50 mm; and over MCI gel CHP20P sorbent (Supelco, USA); TLC, on Sorbfil PTSKh AF-V-UF plates with elution by $CHCl_3$:$(CH_3)_2CO$:HCO_2H:H_2O (5:10:2:1). Spots were detected in UV light or by *p*-nitroaniline/$NaNO_2$ in HCl (yellow or brown color with phenolic compounds) [26].

Preparative RP HPLC was carried out over Diasorb-130-C16T sorbent (7 μm, ZAO BioKhimMakST) in a column (0.7 × 25 cm) with gradient elution by CH_3OH:H_2O from 2 to 100%; HPLC analysis of the products, over the Milikhrom A-02 liquid chromatograph microcolumn using Method 1.

We used *H. theinum* roots collected by the LLiPBAS expedition of the NIOKh SB RAS as previously reported [1]. The preparation of the ethanol extract of the roots and its separation into fractions containing oPA have been previously described [1]. The yield of the acetone fraction was 33%; of the alcohol fraction, 41% of the initial mass of the ethanol extract.

Separation of Acetone Fraction into Narrow oPA Fractions. The acetone fraction (8.61 g) was separated by column chromatography over polyamide (50 g) to afford fraction 1 (1.72 g) by elution with H_2O; fraction 2 (0.46 g) by elution with EtOH:H_2O (35 vol%); fraction 3 (1.75 g) by elution with CH_3OH; and fraction 4 (1.85 g) by elution with $(CH_3)_2CO$:H_2O (70 vol%).

Fraction 3 was separated by column chromatography over MCI gel CHP20P (74 g) by elution with CH_3OH:H_2O from 0 to 100% to afford fraction 3-3 (492 mg, 20% MeOH), fraction 3-4 (147 mg, 35% MeOH), fraction 3-5 (208 mg, 50% MeOH), fraction 3-6 (316 mg, 75% MeOH), and fraction 3-7 (293 mg, 100% MeOH). Fraction 3-3 was separated again by column chromatography over MCI gel CHP20P (26 g) by elution with CH_3OH:H_2O from 0 to 100% to afford fraction 3-2-2 (39 mg, 10% MeOH), 3-2-3 (60 mg, 15% MeOH), 3-2-4 (73 mg, 20% MeOH), 3-2-5 (70 mg, 25% MeOH), 3-2-6 (64 mg, 30% MeOH), 3-2-7 (40 mg, 35% MeOH), 3-2-8 (79 mg, 50% MeOH), and 3-2-9 (33 mg, 100% MeOH).

Fraction 4 was separated by column chromatography over MCI gel CHP20P (74 g) by elution with CH_3OH:H_2O from 20 to 100% to afford fraction 4-1 (804 mg, H_2O), 4-2 (52 mg, 20% MeOH), 4-3 (39 mg, 30% MeOH), 4-4 (291 mg, 50% MeOH), 4-5 (362 mg, 75% MeOH), 4-6 (125 mg, 100% MeOH). Fraction 4-1 was separated again over MCI gel CHP20P (74 g) by elution with CH_3OH:H_2O from 0 to 100% to afford fraction 4-1-3 (24 mg, 20% MeOH), 4-1-4 (28 mg, 30% MeOH), 4-1-5 (93 mg, 30% MeOH), 4-1-6 (171 mg, 30% MeOH), 4-1-7 (250 mg, 40% MeOH), 4-1-8 (55 mg, 40% MeOH), 4-1-9 (61 mg, 50% MeOH), and 4-1-10 (14 mg, 100% MeOH).

Separation of Alcohol Fraction. The alcohol fraction (5.00 g) was separated by column chromatography over polyamide (20 g) to afford fraction sp1 (1.31 g) by elution with water; fraction sp2 (1.32 g) by elution with methanol; and fraction sp3 (0.27 g) by elution with acetone:water (70 vol%).

Thiolysis of oPA Fractions. Solutions of fractions 3-2-2 through 3-7, 4-1-3 through 4-4, sp2, and sp3 (10 mg) in methanol (3 mL), HCl in MeOH (0.15 mL, 0.2 M), and benzylmercaptan (10 μL) were heated under Ar for 5 min at 90°C. The reaction mixtures were analyzed by HPLC without processing using Methods 1 and 2.

Preparative Isolation of Thiolysis Products. A solution of fraction 4-1-7 (230 mg) in methanol (3 mL), glacial acetic acid (1 mL), and benzylmercaptan (0.23 mL) were heated under Ar for 38 h at 100°C with HPLC monitoring. The mixture was evaporated. The solid was dissolved in MeOH (2 mL) and separated by preparative RP HPLC to afford flavan-3-ols **1** (2 mg), **2** (2 mg), **3** (1 mg), **4** (1 mg), and thio-derivatives **5** and **6** (mixture, 9 mg, 1.1:1), **6** (12 mg), **7** (11 mg), **8** (7 mg), and **9** (7 mg).

(3,4-*trans*)-4-Benzylthiogallocatechin (5): UV spectrum (MeOH, λ_{max}, nm): 208, 270. PMR (300 MHz, acetone-d_6, δ, ppm, J/Hz): 3.79 (1H, d, J = 12, SCH_2), 3.95 (1H, d, J = 12, SCH_2), 4.00 (1H, d, J = 6, H-4), 4.17 (1H, m, H-3), 4.42 (1H, d, J = 8, H-2), 5.96 (1H, d, J = 2, H-8), 6.06 (1H, d, J = 2, H-6), 6.46 (2H, s, H-2′,6′), 7.20-7.48 (5H, m, H-2″,3″,4″,5″,6″), 7.97 (5H, br.s, OH). Table 1 lists the ^{13}C NMR spectrum.

(3,4-*cis*)-4-Benzylthiogallocatechin (6): UV spectrum (MeOH, λ_{max}, nm): 210, 270. PMR spectrum (500 MHz, acetone-d_6, δ, ppm, J/Hz): 3.88 (1H, br.d, $J_{OH\text{-}3,H\text{-}3}$ = 6.2, OH-3), 4.07 (1H, d, J = 12.4, SCH_2), 4.11 (1H, ddd, $J_{H\text{-}3,H\text{-}2}$ = 9.6, $J_{H\text{-}3,OH\text{-}3}$ = 6.2, $J_{H\text{-}3,H\text{-}4}$ = 4.3, H-3), 4.12 (1H, d, J = 12.4, SCH_2), 4.37 (1H, d, $J_{H\text{-}4,H\text{-}3}$ = 4.3, H-4), 4.87 (1H, d, $J_{H\text{-}2,H\text{-}3}$ = 9.6, H-2), 5.81 (1H, d, $J_{H\text{-}8,H\text{-}6}$ = 2.3, H-8), 6.01 (1H, d, $J_{H\text{-}6,H\text{-}8}$ = 2.3, H-6), 6.51 (2H, s, H-2′,6′), 7.20 (1H, tt, $J_{H\text{-}4″,H\text{-}3″(5″)}$ = 7.3, $J_{H\text{-}4″,H\text{-}2″(6″)}$ = 1.3, H-4″), 7.28 (2H, dd, $J_{H\text{-}3″,H\text{-}2″}$ = $J_{H\text{-}5″,H\text{-}6″}$ = 7.6, $J_{H\text{-}3″,H\text{-}4″}$ = $J_{H\text{-}5″,H\text{-}4″}$ = 7.3, H-3″, H-5″), 7.41 (2H, br.d, $J_{H\text{-}2″,H\text{-}3″}$ = $J_{H\text{-}6″,H\text{-}5″}$ = 7.6, H-2″,H-6″), 7.83 (3H, br.s, 3OH), 8.07 (1H, br.s, OH), 8.28 (1H, br.s, OH). Table 1 lists the ^{13}C NMR spectrum.

(3,4-*trans*)-4-Benzylthioepigallocatechin (7): UV spectrum (MeOH, λ_{max}, nm): 210, 270. PMR spectrum (300 MHz, acetone-d_6, δ, ppm, J/Hz): 3.89 (1H, br.s, OH-3), 4.01 (1H, m, H-3), 4.08 (1H, d, J = 12, SCH_2), 4.09 (1H, d, J = 12, SCH_2), 4.09 (1H, d, J = 2, H-4), 5.22 (1H, br.s, H-2), 5.91 (1H, d, J = 2, H-8), 6.03 (1H, d, J = 2, H-6), 6.57 (2H, s, H-2′,6′), 7.20-7.50 (5H, m, H-2″,3″,4″,5″,6″), 8.05 (5H, br.s, OH). Table 1 lists the ^{13}C NMR spectrum.

(3,4-*cis*)-4-Benzylthiocatechin (8): UV spectrum (MeOH, λ_{max}, nm): 278. PMR spectrum (300 MHz, acetone-d_6, δ, ppm, J/Hz): 3.95 (1H, br.s, OH-3), 4.11 (1H, d, J = 12, SCH_2), 4.13 (1H, d, J = 12, SCH_2), 4.17 (1H, m, H-3), 4.40 (1H, d, J = 4, H-4), 4.96 (1H, d, J = 10, H-2), 5.84 (1H, d, J = 2, H-8), 6.04 (1H, d, J = 2, H-6), 6.83 (2H, br.s, H-5′,6′), 6.96 (1H, br.s, H-2′), 7.19-7.47 (5H, m, H-2″,3″,4″,5″,6″), 8.20 (4H, br.s, OH). Table 1 lists the ^{13}C NMR spectrum.

(3,4-*trans*)-4-Benzylthioepicatechin (9): UV spectrum (MeOH, λ_{max}, nm): 280. PMR spectrum (300 MHz, acetone-d_6, δ, ppm, J/Hz): 3.92 (1H, d, J = 6, OH-3), 4.01 (1H, m, H-3), 4.03 (1H, d, J = 13, SCH_2), 4.04 (1H, d, J = 13, SCH_2),

4.10 (1H, d, J = 2, H-4), 5.28 (1H, br.s, H-2), 5.92 (1H, d, J = 2, H-8), 6.04 (1H, d, J = 2, H-6), 6.80 (2H, m, H-5′,6′), 7.05 (1H, d, J = 1.5, H-2′), 7.20-7.50 (5H, m, H-2″,3″,4″,5″,6″), 8.20 (4H, br.s, OH). Table 1 lists the ^{13}C NMR spectrum.

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