A Novel Type of Flavonoids: Flavonol Esters from Fern Exudates

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Farinose exudates on fronds of *Notholaena* species are known to consist of flavonoid aglycones. These are predominantly methylated flavones and flavonols, sometimes chalcones and dihydrochalcones. Flavonols with unusual properties, excreted as major farina components by some species, have been found to be derivatives of methylated flavonols, esterified in position 8 with butyric or acetic acids, respectively. These are a novel type of flavonoids. They occur as twin-pairs, some with very great differences in proportions. Their presence in a group of species within the genus has chemotaxonomic implications. Two of the flavonols resulting from hydrolysis are new compounds.

Quite recently several reports appeared dealing with the chemical composition of the farinose deposit on the under surface of fronds of ferns belonging to the genera *Cheilanthes*, *Notholaena* and *Pityrogramma* (fam. Polypodiaceae, subfam. Gymnogrammoideae). In *Pityrogramma* chalcones and dihydrochalcones predominate [1], whereas in the two other genera they are found rather rarely [2]. *Cheilanthes* and *Notholaena* produce mainly more or less lipophilic methyl ethers of flavones and flavonols [3]. It has been mentioned previously [3] that some samples of *N. affinis* (Mett.) Moore and *N. californica* D. C. Eaton had been found to show "completely unknown, but most probably flavonoid compounds". Thanks to the support of some pteridologists supplying material for these investigations in sufficient amount, it has been possible now to isolate at least the major components. From the farina of *N. affinis* we identified novel 2′-substituted flavonols with high degree of methylation of ring A [4, 5]. Most interesting, however, are the esterified flavonols encountered in several species of *Notholaena*. Here we report on elucidation of their structures.

Materials and Methods

Fern material was collected in the following places (collector's names and voucher numbers in brackets): *N. affinis* (L. D. Gómez 4725) in Costa Rica, *N. aschenborniana* Kl. (D. J. Pinkava & T. Reeves R 4315 and R 4316) and *N. neglecta* Maxon (D. J. Pinkava & T. Reeves R 4310B and R 4320) in Mexico, *N. californica* (T. Reeves R 5353) in California, *N. galapagensis* Weath. & Svenson (A. & H. Adsersens No. 1972) on the Galapagos Islands. The powdery flavonoid layer can be dissolved by rinsing the fronds with acetone and benzene at room temperature, sometimes with addition of methanol. The compounds investigated are in part major components of the farina and were precipitated from concentrated extracts. They were purified by repeated crystallisation from methanol. *NA-2* and *NA-3* were isolated from the residue of *N. affinis* (after crystallisation of *NA-1*) by column chromatography and preparative thin layer chromatography. For CC we used Polyamid SC-6 (Macherey & Nagel); elution was with benzene and increasing amounts of methylethylketone and methanol. Preparative TLC: Separation of *NG-1* and *NG-2* as well as of *NA-1* and *NA-2* was performed by TLC on Polyamid DC-11 with solvent A, or on silica gel with solvent benzene/dioxan/MeOH 90 : 12 : 3. Also *NAS-1* and *NAS-2* were separated and *NA-3* was isolated by preparative TLC on silica gel. Solvents for polyamide: A) benzene/petrol$_{100-140°}$/methylethylketone/methanol 30 : 60 : 5 : 5 or B) 60 : 26 : 7 : 7 or C) benzene/methylethylketone/methanol 60 : 26 : 14. Thin layer chromatograms were evaluated in longwave UV-light, before and after spraying with "Naturstoffreagenz A" (β-aminoethyl ester of diphenyl boric acid). Acid saponification: A solution of the substance in a small amount of boiling glacial acetic acid was treated with some drops of con-

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centrated hydrochlorid acid. The reaction product precipitated while the solution was cooling down. Total demethylation [6]: 2 mg of the substance were mixed with 20 mg pyridine-HBr and heated on an oil bath at 250 °C for 5 minutes. After cooling the reaction mixture was treated with water and acetone and extracted with ether. Mass spectra were recorded using a Varian MAT 311 A at the institute of organic chemistry of the Technische Hochschule Darmstadt, PMR-spectra were recorded using a Varian XL 100 and peak-matching was performed using an AEI MS 902 at the Université Claude Bernard Lyon I.

Results

All the compounds dealed with here are characterised by the fact that they rather easily precipitate from concentrated solutions, although they are different in polarity (see Table I). They crystallise as light yellow needles. On polyamide layers they appear orange-yellow in UV-light, *e. g.* just like flavonols kaempferol and quercetine. None of them is identical, however, with one of the many reference substances at our disposal. After spraying with "Naturstoffreagenz A" they do not turn greenish-yellow, but rather fluoresce yellow. Also their molecular weights are not in accordance with the structures of "normal", *i. e.* OH- and OCH_3-substituted flavonoids. Hence they must be compounds with unusual substituents. The reaction products obtained on treatment with hydrochloric acid are more polar than the original products. This leads to the assumption that they are esters.

NG

This "substance", observed at first in the farina of *N. californica*, after fruitless efforts to obtain bulk material of this species, finally was isolated from the exudate of *N. galapagensis*. On TLC in non-polar solvents it can be seen that in reality it is a "twin-pair", the components of which can be separated only by preparative TLC. The substance causing the lower spot (*NG-2*) could be isolated from the farina of *N. neglecta* as a major constituent.

Two peaks at *m/e* 370 and 342, observed in the mass spectrum of *NG*, appear as M^+ in the mass

Table I. R_f-values and UV-spectra of the flavanol-esters and the products of saponification ("sap.").

Substance	$R_f \times 100$ Solvent A	B	C	UV-spectra (λ_{max} [nm]) in MeOH	+NaOAc	+NaOAc +H_3BO_3	+$AlCl_3$	+$AlCl_3$ +HCl	+NaOH
NG-1	62	83	—	366, 266	374, 266	368, 268	426, 334, 274	426, 334, 274	418, 268
NG-2	51	78	—						
NG-sap.	12	31	78	390, (302), 280	375, 271	375, 272	450, 350, (315), 268, 246	450, 346, (313), 283, 246	370, 268
NAS-1	8	25	(88)	375, 324, 270	384, 268	376, 268	438, 356, (310), 270	440, 354, 268	426, 344, 268, 250
NAS-2	5	19	85						
NAS-sap.	—	4	39	388, 332, 277	383, 276	380, 336, 276	452, 365, 316, 283, 268	452, 365, 320, 282, 266	376, 284, 250
NA-1	48	81	—	373, (320), 268	(408), 384, (318), 268	372, (318), 268	435, 355, (308), 268	435, 353, (307), 268	418, 264 etwas instabil
NA-2	43	77	—						
NA-sap.	8	26	77	388, 330, (308), 276	385, (320), 276	380, (342), 277	448, 369, 285, 270	450, 362	366, 248 instabil
NA-3	13	41	(91)	378, (330), (304), (270), 257	(420), 392, 258	378, (290), (270), 256	438, 362, 266	436, 360, 267	422, 268

spectra of *NG-1* and *NG-2*, respectively; *m/e* 300 as the base-peak and lower fragments are identical in both spectra. Here we provide data only on the product of saponification. *m/e* 300 corresponds with a "normal" flavone or flavonol with 3 hydroxy groups and 1 methoxy group.

The UV-spectra of both substances are completely identical as are the reactions to the classical reagents [7]. The reaction with $AlCl_3$ and $AlCl_3$/HCl confirm what had been assumed by the colour development on polyamide: they are flavonols with free OH-groups at C-3 and C-5. Furthermore the spectrum in MeOH points to a flavonol of the galangine type [7], *i.e.* with an unsubstituted B-ring. Position 7 is blocked; presumably the O-methyl group is located here. The PMR-spectrum indicates an unsubstituted B-ring, too, and this is indicated also by the presence of *m/e* 105 ("Pic C" [8]) in the mass spectrum. Besides we observe a signal for H-6, but not for H-8. The PMR-spectrum furthermore shows the presence of a terminal $-CO-CH_3$ group. Fragmentation of M-42 (*NG-2*) is in accordance with loss of an acetyl unit; M-70 (*NG-1*) corresponds to loss of a butyryl unit. Indeed on acid hydrolysis of *NG-1* the smell of butyric acid can be detected. Determination of the molecular weights by high resolution mass spectroscopy leads to 370.1061 for *NG-1* (calculated for $C_{20}H_{18}O_7$, 370.1052) and 342.0739 for *NG-2* (calculated for $C_{18}H_{14}O_7$, 342.0739) and 300.0634 for the hydrolysed compound (calculated for $C_{16}H_{12}O_6$, 300.0645).

The aglycone crystallises from the reaction mixture of acid hydrolysis in fine yellow needles, m.p. 246 °C. On polyamide TLC it forms a weak brownish spot at much lower R_f than the original compounds, which turns reddish brown on spraying with "Naturstoffreagenz A". Again the PMR spectrum shows an unsubstituted B-ring, a proton at C-6 and one methyl group. The UV-spectrum indicates a free hydroxy group at C-8 (bathochromic shift of λ_{max}^{MeOH} 24 nm, compared to *NG*).

MS *m/e* (rel.int.): 300 (100), 285 (13), 271 (8), 269 (2), 257 (14), 183 (2), 167 (2), 155 (3), 153 (3), 139 (13), 111 (5), 105 (19), 77 (18). With regard to all the data available this product must be the 7-methylether of 8-hydroxy-galangin.

Demethylation yields a product of still lower R_f. It appears as a brown spot on polyamide, which becomes dark after spraying (bluish in daylight). The solution after addition of ethylate turns blue – a reaction which recalls the "gossypetin-test" [9], a hint for the 3,5,7,8-OH-substitution pattern.

Considering all the analytical results, *NG-1* is the butyric acid ester and *NG-2* is the acetic acid ester of 8-hydroxy-galangin-7-methyl ether.

NAS

Here we also have a mixture of two components, isolated from the farina of *N. aschenborniana*. These compounds are similar in behaviour to *NG-1* and *NG-2*, but have much lower R_f values. Again the UV-spectra for both substances are identical. The arguments for structure elucidation are about the same as for *NG* and hence may be reported rather briefly. Differences are in higher molecular weights (386 and 358, base-peak at *m/e* 316) and "Pic C" [8] at *m/e* 121, behaviour of the UV-spectrum with alkali (comp. Table I) and in the presence in PMR-spectrum of the AA′BB′ spin system typical of *p*-substituted aromatic ring. From these observations it can be concluded that the B-ring has an OH-group at C-4′. So we probably have the esterified 7-methyl ether of 8-hydroxy-kaempferol. Peak-matching results in a mass of 386.0999 for *NAS-1* (calculated for $C_{20}H_{18}O_8$, 386.1002) and 358.0695 for *NAS-2* (calculated for $C_{18}H_{14}O_8$, 357.0689), 316.0680 for the aglycone (calculated for $C_{16}H_{12}O_7$, 316.0583).

Acid hydrolysis of *NAS* yields a yellow crystalline compound, m.p. 285 °C (dec.). The maxima in UV-spectrum differ by a few nms from those cited in the literature [10]; there is no doubt, however, that this product is identical with herbacetin-7-methyl ether. MS *m/e* (rel.int.): 3160 (100), 301 (20), 287 (6), 285 (2), 273 (11), 259 (3), 183 (4), 167 (3), 153 (3), 139 (10), 121 (17), 93 (5). Demethylation of the saponification product yields a more polar compound. This appears as a brownish spot on polyamide, becoming dark after spraying (bluish-violet in daylight). Here also the solution on addition of ethylate turns blue. Direct comparison with an authentic sample of herbacetin confirms the identity of both substances.

NAS-1 is the butyric acid ester, *NAS-2* is the acetic acid ester of herbacetin-7-methyl ether.

NA

The substance *NA-1* has been isolated as a major product from the farina of *N. affinis*. Elucidation

Table II. PMR-spectra of some flavonol esters and of flavonols obtained by saponification ("sap."). (100 MHz, DMSO-D-6; δ ppm/TMS).

Flavonol esters	Flavonols
NG-2	*NG*-sap.
2.40 3 H *s* CO−CH_3	3.91 3 H *s* −OCH_3
3.93 3 H *s* −OCH_3	6.54 1 H *s* H−6
6.68 1 H *s* H−6	7.58 3 H *m* H−3′4′5′
7.60 3 H *m* H−3′4′5′	8.23 2 H *dd* H−2′6′ (*J* 8.5 and 2.5 Hz)
8.06 2 H *dd* H−2′6′ (*J* 8.5 and 2.5 Hz)	*NAS*-sap.
	3.92 3 H *s* −OCH_3
	6.56 1 H *s* H−6
	6.95 2 H *dd* H−3′5′ (*J* 8.5 and 2.5 Hz)
	8.15 2 H *dd* H−2′6′ (*J* 8.5 and 2.5 Hz)
NA-1	*NA*-sap.
1.02 3 H *t* −CH_3 (*J* 7 Hz)	3.88 3 H *s* −OCH_3
1.75 2 H *sext.* −CH_2− (*J* 7 Hz)	3.93 3 H *s* −OCH_3
2.70 2 H *t* CO−CH_2− (*J* 7 Hz)	6.59 1 H *s* H−6
3.85 3 H *s* −OCH_3	7.16 2 H *d* H−3′5′ (*J* 9 Hz)
3.91 3 H *s* −OCH_3	8.27 2 H *d* H−2′6′ (*J* 9 Hz)
6.66 1 H *s* H−6	
7.10 2 H *d* H−3′5′ (*J* 9 Hz)	
8.03 2 H *d* H−2′6′ (*J* 9 Hz)	
NA-3	
1.00 3 H *t* −CH_3 (*J* 7 Hz)	
1.74 2 H *sext.* −CH_2− (*J* 7 Hz)	
2.72 2 H *t* CO−CH_2− (*J* 7 Hz)	
3.85 3 H *s* −OCH_3	
3.89 3 H *s* −OCH_3	
6.66 1 H *s* H−6	
7.11 1 H *d* H−5′ (*J* 8.5 Hz)	
7.56 2 H *m* H−2′6′ (*J* 8.5 and 2.5 Hz)	

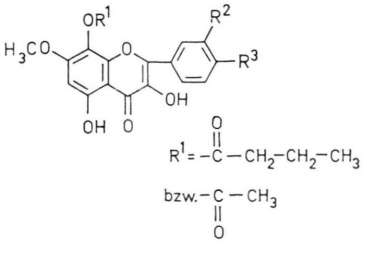
of its structure as 8-butyryl ester of herbacetin-7,4′-dimethyl ether has been reported recently [11]. In Tables I and II the data of this compound are included to allow direct comparison with the analogous compounds. From the remaining of the extract from the isolation of the initially mentioned polymethoxy-flavonols it was possible to isolate a very small amount of the related substance *NA-2*. This was not sufficient for a PMR-spectrum. All other findings, however, above all in MS (M^+ 372, base peak 330), allow its identification as the acetyl compound corresponding to *NA-1*.

The product of saponification had not been described in reference [11]. It crystallises in yellow needles, m.p. 226 °C. Evaluation of the UV-spectrum confirms once more the proposed structure as 7,4′-dimethyl ether of herbacetin. MS *m/e* (rel.int.): 330 (100), 315 (46), 301 (12), 300 (6), 299 (4), 287 (14), 153 (4), 135 (24). Certainty comes from complete demethylation, which again yields herbacetin.

When working up the remainder of *N. affinis* extracts a further yellow spot was observed on TLC. A small amount of the product could be isolated by preparative TLC on silica gel. The molecular weight of 416 (base-peak at *m/e* 346 = M^+-70) indicates the presence of an additional OH-group, compared with *NA-1*/*NA-2*. The compound also is more polar. Interpretation of the mass spectrum and of the UV-spectrum and the PMR-spectrum shows that this product *NA-3* is the butyryl ester of gossypetin-7,4′-dimethyl ether. In the mass spectrum a small peak is observed at *m/e* 388, which indicates the presence of the corresponding acetyl ester, *NA-4*. Saponification and demethylation could not be performed in this case because of lack of material.

Discussion

The investigations presented here have shown that certain *Notholaena*-species are able to exude not only flavonoid aglycones but also esterified flavonoid aglycones. Hitherto we found monoesters with butyric acid and with acetic acid, respectively. All the compounds of this kind are flavonols with a



NG : R^2=R^3=H
NAS: R^2=H , R^3=OH
NA-½: R^2=H , R^3=OCH_3
NA-¾: R^2=OH , R^3=OCH_3

methoxy group at C-7, esterified at C-8. The products differ by substitution of ring B.

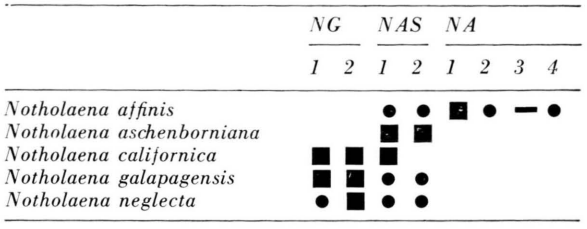
These very stable butyric acid esters are the first representatives of a new type of flavonoids. Substitution with an isoprenoid side-chain occurs rather frequently [12]; its formation is biogenetically evident. The butyryl side-chain is present in phloroglucinols from the fern genus *Dryopteris* [13], but there it is C-C-linked. Recently an O-prenylated dihydrochalcone has been reported from *Lonchocarpus neuroscapha* [14] and a new isoflavone with O-3-methyl-2-enyl substitution has been isolated from seeds of *Milletia auriculata* [15]. True esters with butyric acid, however, have not been reported heretofore.

Esters of acetic acid already found in nature are 3-acetate of pinobanksin from bud exudate of *Populus* species [16], and its 7-methyl ether from seeds of *Alpinia japonica* [17], and 2-methyl-7-hydroxy-8-acetyl isoflavone reported recently to occur in roots of *Glycyrrhiza glabra* [18].

Among the flavonol aglyca found here as esters the gosspetin-7,4′-dimethyl ether has been known only from seeds of *Xanthoxylum acanthopodium* [19].The herbacetin-7-methyl ether "pollenitin" has been reported from pollen of *Camellia sinenesis* [10] and from leaves of *Athraphaxis pyrifolia* [20] (presumably glycosides in every case). The 7,4′-dimethyl ether of herbacetin and the 7-methyl ether of 8-hydroxy-galangin to our knowledge are new natural flavonols.

The following survey shows the distribution and relative amounts of flavonol esters present in farina of the ferns analysed here.

These statements are valid only for the collections cited here with herbarium numbers, *i. e.* for certain



populations and must not be generalised. We have good reason to assume the existence of chemotypes with, in part, totally different flavonoid patterns [3]. Butyryl esters and acetyl esters obviously always occur jointly, although in very variable amounts.

The study of farinose species of the genus *Notholaena* indicates that there are additional species which are able to produce the novel flavonol esters described here (Wollenweber, unpubl. results). After further investigation of more specimens their distribution will be reported in detail. The assumption is that there exists a certain taxonomic grouping within the genus which is characterised by this biosynthetic capability.

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[1] E. Wollenweber, Z. Pflanzenphysiol. **78,** 344 (1976).
[2] E. Wollenweber, Z. Naturforsch. **32 c,** 1013 (1977).
[3] E. Wollenweber, Z. Pflanzenphys. **85,** 71 (1977).
[4] M. Jay, E. Wollenweber, and J. Favre-Bonvin, Phytochemistry, in press.
[5] M. Jay, J. Favre-Bonvin, and E. Wollenweber, Canad. J. Chem., in press.
[6] G. Howard and T. J. Mabry, Phytochemistry **9,** 2413 (1970).
[7] M. Jay, J.-F. Gonnet, E. Wollenweber, and B. Voirin, Phytochemistry **14,** 1605 (1975).
[8] H. Audier, Bull. Soc. Chim. France **1966,** 2892.
[9] J. B. Harborne, Phytochemistry **8,** 177 (1969).
[10] Y. Sakamoto, Agr. Biol. Chem. **33,** 818 (1969).
[11] E. Wollenweber, J. Favre-Bonvin, and P. Lebreton, Phytochemistry **17,** 1684 (1978).
[12] K. Venkataraman, The Flavonoids (J. B. Harborne, T. J. Mabry, and H. Mabry, Eds.), p. 277. Chapman and Hall, London 1975.
[13] C. J. Widén, J. van Euw, and T. Reichstein, Helv. Chim. Acta **53,** 2176 (1970).
[14] A. Lupi, G. Delle Monache, F. Delle Monache, and G. B. Marini-Bettolo, Farmaco Ed. Sci. **30,** 449 (1975).
[15] K. V. Subba Raju and G. Srimannayarana, Phytochemistry **17,** 1065 (1978).
[16] E. Wollenweber, Z. Naturforsch. **28 c,** 638 (1973).
[17] J. Gripenberg and K. Silander, Chem. Ind. **1955,** 443.
[18] D. K. Bhardwaj, T. R. Seshadri, and R. Singh, Phytochemistry **16,** 402 (1977).
[19] J. B. Harborne, P. Lebreton, H. Combier, T. J. Mabry, and Z. Hamman, Phytochemistry **10,** 883 (1971).
[20] T. K. Chumbalov, M. M. Mukhmahed'yarova, and V. B. Omurkamzinova, Khim. Prir. Soedin. **1974,** 793.