# 108. Isolation and Structure Elucidation of Some Components of the Antitumor Antibiotic Mixture 'Rubiflavin'[1])

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The antitumor antibiotic 'rubiflavin' was investigated. It was shown to be a mixture of several compounds, nine of which – after isolation by HPLC – could be identified by $^1$H-NMR spectroscopy. The rubiflavins A (4), B (5), C-1 (6), C-2 (7), D (8), and E (9) are pluramycin antibiotics differing only in their side chains at C(2). Rubiflavin B (5) was found to be identical with kidamycin, rubiflavin F (10) with isokidamycin. Two unpolar compounds isolated which lack the two sugar rings typical for pluramycin antibiotics were called rubiflavinone C-1 (2) and C-2 (3); they are the 'aglycones' of the corresponding rubiflavins.

**Introduction.** – In 1964, *Aszalos et al.* isolated a new antitumor-active antibiotic from a *Streptomyces* species [3]. The substance which was called 'rubiflavin'[2]) was purified by countercurrent distribution and thought to be uniform. The elemental analysis led to the molecular formula $C_{23}H_{29-31}NO_5$. This was in accord with molecular-weight determinations which yielded 386–430 (titration) and 412 (ultracentrifugation). The structure of this 'rubiflavin' was not determined thereafter, but the antibiotic was intensely studied with respect to its biological activities. The most prominent of these were – besides the antitumor activity – the ability to bind to DNA [4] [5] and subsequently inhibit the DNA synthesis [5] [6].

The UV and IR data that *Aszalos et al.* determined for 'rubiflavin' were very similar to those of hedamycin (1), kidamycin (5), and related compounds [7] and thus indicated that 'rubiflavin' probably belonged to the group of pluramycin antibiotics[3]). The $^{13}$C-NMR spectrum of a sample of crude 'rubiflavin' obtained from *Aszalos et al.* corroborated this suggestion; it displayed all the well known resonances of the two 'sugar' rings E and F as in the corresponding spectra of hedamycin (1) and kidamycin (5) [9]. Thus, in 'rubiflavin', these two rings are not acetylated. Accordingly, no ester-carbonyl resonances were detected in the IR spectrum. In the sp$^2$ region of the $^{13}$C-NMR spectrum of 'rubiflavin', the resonances also corresponded more or less to those commonly observed for pluramycin-like antibiotics [9]. However, signal clusters were detected here as well as in the methyl region of the spectrum, suggesting that 'rubiflavin' was a mixture of several pluramycin-like compounds differing only in their side chains at C(2).

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[1])　Taken in part from the dissertation of *H. N.* [1]; for a preliminary account, see [2].

[2])　'Rubiflavin' – in inverted commas – will be used in this paper to denote the samples described by or obtained from *Aszalos et al.* [3].

[3])　The numbering scheme used in the formulae corresponds to that established for pluramycin-like antibiotics in earlier papers [7] [8].
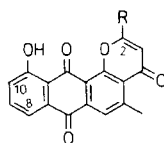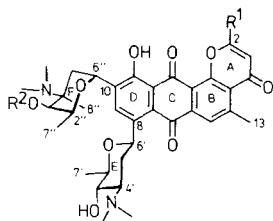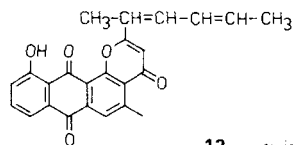
|   |   | R¹ | R² |
|---|---|---|---|
| 1 | hedamycin |   | H |
| 4 | rubiflavin A |   | H |
| 11 | pluramycin A |   | Ac |
| 5 | rubiflavin B, kidamycin |   | H |
| 6 | rubiflavin C-1 |   | H |
| 7 | rubiflavin C-2 |   | H |
| 8 | rubiflavin D |   | H |
| 9 | rubiflavin E |   | H |



|   |   | R |
|---|---|---|
| 2 | rubiflavinone C-1 |   |
| 3 | rubiflavinone C-2 |   |
| 13 | β-indomycinone |   |
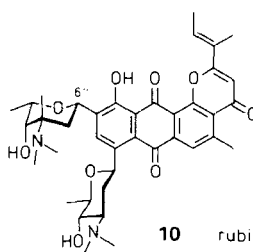


10    rubiflavin F, isokidamycin



12    α-indomycinone

**Isolation of the Components.** – Attempts to separate the 'rubiflavin' mixture into its components using classical column chromatography or prep. TLC with a variety of adsorbents and solvents failed. Therefore, HPLC was used. But even here the separations were so demanding that repetitive runs with very small amounts of material on analytical columns had to be used to get at least 0.5–2.5 mg of reasonably pure material of each component. The following procedure proved to be successful *(cf. Fig. 1)*. First, polar substances of the 'rubiflavin' mixture were removed by chromatography on *Sephadex LH 20* with $CH_2Cl_2$. The polar constituents which remained on the column under these conditions could be eluted using $CH_2Cl_2$ with increasing amounts of MeOH. These polar substances were, however, not investigated further. The unpolar material (*ca.* 61%) was then separated by HPLC on an analytical silica-gel column using $CH_2Cl_2$/MeOH/aq. $NH_3$ mixtures. A maximum of 1 mg of the prepurified unpolar mixture was applied per run. Five fractions were taken *(cf. Fig. 2)*. The third which was the largest (21% with respect to crude 'rubiflavin') contained components whose retention times corresponded more or less to that of hedamycin (**1**).
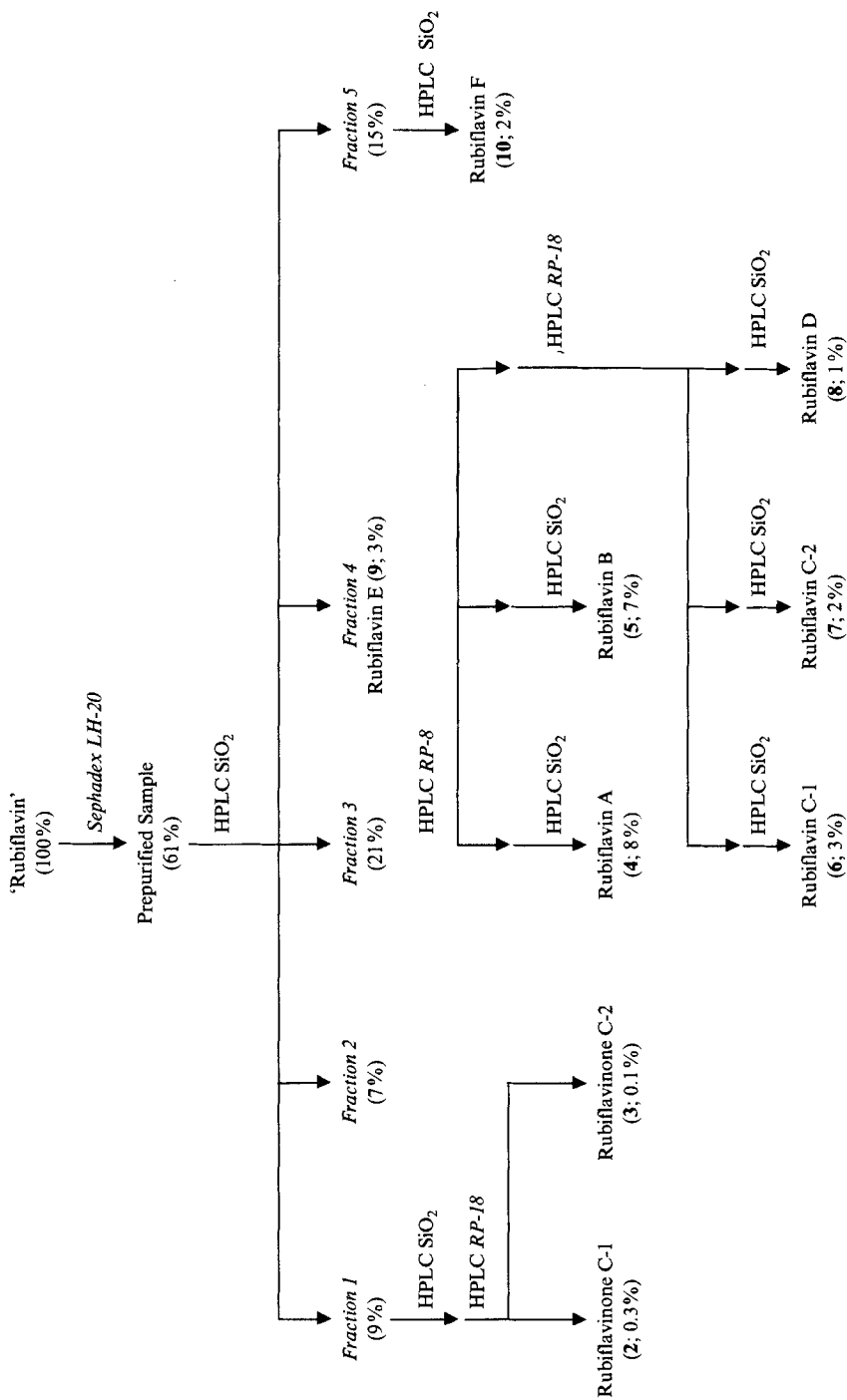
Fig. 1. *Separation scheme for the isolation of the 'rubiflavin' components*
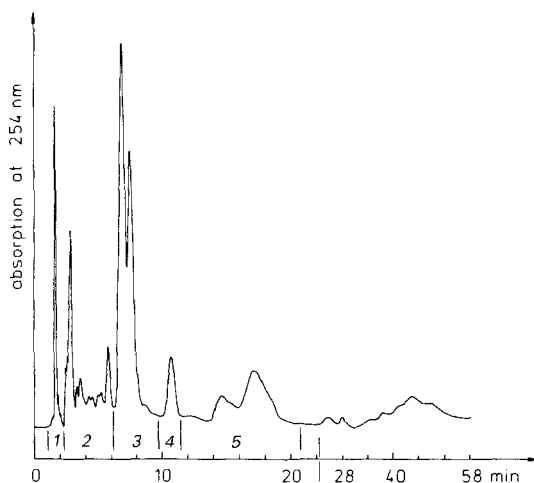
Fig. 2. *HPLC of the 'rubiflavin' mixture. LiChrosorb Si 60 7μ, $CH_2Cl_2/CH_3OH$/aq. $NH_3$ 935:65:4.5; the fractions collected in preparative runs are labelled with 1–5*

*Fraction 1* was re-chromatographed on silica gel with $CH_2Cl_2$ and then on *RP-18* material using MeOH. Two pure compounds were obtained. A preliminary spectral investigation showed that they did not contain the usual sugar rings common to all pluramycin antibiotics but nevertheless were closely related to the rubiflavins C-1 and C-2 from *Fraction 3* (see below); they were, therefore, called rubiflavinones C-1 (**2**) and C-2 (**3**), respectively.

*Fraction 2* contained several compounds in small amounts and was not investigated further.

The $^1$H-NMR spectrum of *Fraction 3* showed that the signal corresponding to H–C(3) was a cluster of 3 resonances thus indicating that this fraction still was a mixture of at least 3 compounds. It could, however, not be separated on silica gel. Reversed phase ion pair chromatography proved suitable for the further separations. Three main components in a ratio of 40:30:25 were eluted with $MeOH/H_2O/PIC$ *B-7* mixtures and were called rubiflavins A (**4**), B (**5**), and C in the order of their elution. The rubiflavins A and B (8 and 7% of the crude antibiotic mixture, resp.) proved to be uniform according to their NMR spectra, whereas rubiflavin C still was a mixture of *ca.* 3 compounds. These could be separated on reversed-phase HPLC columns using $MeCN/H_2O/PIC$ *B-5* mixtures. The 2 compounds first eluted were stereoisomers according to their $^1$H-NMR spectra and were called rubiflavins C-1 (**6**) and C-2 (**7**), respectively (3 and 2% of the crude 'rubiflavin'), whereas the third component was named rubiflavin D (**8**; 1%). The substances obtained from the ion-pair chromatography were first freed from the bulk of the *PIC* reagent by partition between $CHCl_3$ and aq. $NaHCO_3$ solution; then the last 1–1.5 equiv. of *PIC* reagent were removed by HPLC on silica gel.

According to its $^1$H-NMR spectrum, *Fraction 4* proved to be a single compound (3% of the original mixture) which was called rubiflavin E (**9**).

After re-chromatography, *Fraction 5* yielded besides several minor compounds one major product which was called rubiflavin F (**10**; 2% of the crude mixture).

**Structure Elucidations.** – Since only small amounts (*ca.* 0.5–2.5 mg) of compounds **2–10** were available, structural elucidation was mainly based on ¹H-NMR spectroscopy *(cf. Table)*. The task was facilitated inasmuch as the resonances of the 4*H*-anthra[1,2-*b*]pyran-4,7,12-trione system and of the two amino-sugar rings were well known from the detailed studies of hedamycin (**1**) and kidamycin (**5**) [7]. Thus, the rubiflavins A, B, C-1, C-2, D, and E (**4–9**) were readily recognized as pluramycin-like antibiotics; the corresponding resonances were identical with those of the references **1** and **5** with respect to chemical shift and multiplicity *(cf. Table)*. The main problem, therefore, was the elucidation of the nature of the side chain at C(2) of the different compounds which was solved by homonuclear-decoupling and NOE-difference ¹H-NMR experiments. The molecular weights of the compounds isolated could be determined by MS and served as the starting points for these investigations.

The ¹H-NMR spectrum of rubiflavin A (**4**) indicated that its side chain contained a $CH_3$–CH=CH fragment and probably a trisubstituted epoxide. Homonuclear decoupling experiments confirmed these presumptions. The coupling constant measured between the olefinic protons was 10.5 Hz pointing to the (*Z*)-configuration for the double bond. Rubiflavin A was the only compound of which sufficient material was at hand for measuring the ¹³C-NMR spectrum [2]. This spectrum fully corroborated the structural features derived so far. The resonances of the aromatic and of the two sugar moieties were identical with the corresponding signals of hedamycin (**1**) [9]. The resonances of the side-chain C-atoms were observed at 134.0 and 123.3 ppm (C(18) and C(17), double bond), at 61.1 and 59.1 ppm (C(16), C(14), epoxide), and at 14.5 and 13.8 ppm (C(15), C(19), $CH_3$ groups). The chemical shifts of both these side-chain $CH_3$ groups were at rather high fields, so both $CH_3$ had to experience an upfield shift due to a synperiplanar $\gamma$-group (*cf.* [10]). This corroborated the (*Z*)-configuration of the double bond derived from the ¹H-NMR spectrum and lead to the epoxide configuration indicated in **4**. NOE-difference spectra further supported these configurational assignments. When the protons at C(15) were irradiated, the resonance of H–C(17) showed a distinct enhancement; on the other hand, irradiation at the $CH_3$(19) signal gave an NOE for H–C(16). In addition, the ¹H-NMR spectrum of the triacetyl derivative of **4** was virtually identical with that of di-*O*-acetylpluramycin A [11]. Hence, rubiflavin A (**4**) was shown to be the desacetyl derivative of pluramycin A (**11**) [10–12]. The configurations at C(14) and C(16) relative to those in rings E and F could not be determined. Recently, *Gonda et al.* reported that rubiflavin A was also one of the chromophore constituents of the protein antitumor antibiotic largomycin FII [13].

The side chain in rubiflavin B (**5**) had the 1-methyl-1-propenyl constitution according to the NMR spectra. Thus, rubiflavin B is identical with kidamycin (**5**) [8]. Indeed, comparison with an authentic sample of kidamycin showed the chromatographical behaviour and the spectra of the two compounds to be identical as were those of the corresponding two triacetyl derivates.

The ¹H-NMR spectrum of rubiflavin C-1 (**6**) revealed the signals of 3 vinyl protons at 8.39, 6.54, and 6.10 ppm as well as of 2 $CH_3$ groups at 2.19 and 2.08 ppm. The former overlapped, however, with the signal of one of the dimethylamino groups; separated resonances could be observed when the spectrum was measured at higher temperature and at 400 MHz. Decoupling experiments showed that a 1-methyl-1,3-pentadienyl side chain had to be present, reminiscent of the side chain that *Brockmann et al.* had found in α-indomycinone (**12**) [14]. The coupling constant of 10.5 Hz for H–C(17) and H–C(18) indicated a (*Z*)-configuration; the one observed between H–C(16) and H–C(17) (11.5 Hz) corresponded to what is usually observed for the central bond in a conjugated diene with s-*trans*-conformation [15]. According to its ¹H-NMR spectrum, rubiflavin C-2 (**7**) was closely related to rubiflavin C-1 (**6**). Again, the signals of 3 vinyl protons (7.94, 6.59, and 6.39 ppm) and 2 $CH_3$ groups (2.00 and 2.09 ppm) could be discerned, and decoupling experiments proved the 1-methyl-1,3-pentadienyl constitution of the side chain in **7**. However, in contrast to **6**, the coupling constant for H–C(17) and H–C(18) was 15 Hz, indicating an (*E*)-configuration; *J*(16, 17) was again 11.5 Hz, thus pointing to the s-*trans*-conformation of the diene system. The configuration of the trisubstituted C(14)=C(16) bond in **6** and **7** could not be determined directly, since the very low amounts of material available did not allow NOE-difference experiments. We assume, however, that it is (*E*) as in the corresponding rubiflavinones C-1 (**2**) and C-2 (**3**; see below) since all the side-chain resonances of both rubiflavins **6** and **7** corresponded exactly to those of the respective rubiflavinones with regard to chemical shifts and coupling constants.

The ¹H-NMR spectrum of rubiflavin D (**8**) showed resonances for a vinyl proton at 7.46 ppm, a $CH_2$ group at 1.65 and 2 $CH_3$ groups at 2.01 and 1.08 ppm. The coupling pattern revealed a kidamycin-like CH=C($CH_3$)

Table. $^1$H-NMR Data of Hedamycin (1), the Rubiflavins 4–10, and the Rubiflavinones 2 and 3[3])[a]

| | Hedamycin (1) | Rubiflavin A (4) | Rubiflavin B (5) | Rubiflavin C-1 (6) | Rubiflavin C-2 (7) | Rubiflavin D (8) | Rubiflavin E (9) | Rubiflavin F (10) | Rubiflavinone C-1 (2) | Rubiflavinone C-2 (3) | β-Indomycinone (13)[b] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| H–C(3) | 6.46 (s) | 6.52 (s) | 6.38 (s) | 6.45 (s) | 6.43 (s) | 6.39 (s) | 6.53 (s) | 6.38 (s) | 6.45 (s) | 6.42 (s) | 6.52 (s) |
| H–C(6) | 8.00 (s) | 8.02 (s) | 7.97 (s) | 7.95 (s) | 7.95 (s) | 7.96 (s) | 8.00 (s) | 7.95 (s) | 8.05 (d, J = 0.5) | 8.04 (d, J = 0.5) | 8.03 (s) |
| H–C(8) | – | – | – | – | – | – | – | – | 7.84 (dd, J = 2, 7.5) | 7.84 (dd, J = 2, 8) | 7.79 (q) |
| H–C(9) | 8.32 (s) | 8.34 (s) | 8.31 (s) | 8.32 (s) | 8.32 (s) | 8.32 (s) | 8.33 (s) | 8.40 (s) | 7.69 (t, J = 8) | 7.68 (t, J = 7.5) | 7.65 (t) |
| H–C(10) | – | – | – | – | – | – | – | – | 7.35 (dd, J = 2, 8) | 7.37 (dd, J = 2, 8) | 7.30 (q) |
| HO–C(11) | 14.1 (br. s) | 14.1 (s) | c) | 14.12 (s) | 14.20 (s) | c) | 14.09 (s) | 14.0 (s) | 13.3 (s) | 13.0 (s) | 13.2 (s) |
| CH₃(13) | 2.99 (s) | 3.01 (s) | 3.01 (s) | 3.01 (s) | 3.03 (s) | 3.03 (s) | 2.98 (s) | 3.00 (s) | 3.02 (d, J = 0.5) | 3.02 (d, J = 0.5) | 2.99 (s) |
| CH₃(15) | 1.96 (s) | 1.84 (s) | 2.00 (s) | 2.08 (s) | 2.09 (s) | 2.01 (s) | 1.70 (s) | 2.00 (s) | 2.07 (br. s) | 2.08 (br. s) | 1.69 (s) |
| H–C(16) | 3.33 (d, J = 5) | 4.16 (d, J = 8) | 7.46 (br. q, J = 7) | 8.39 (br. d, J = 11.5) | 7.94 (br. d, J = 11.5) | 7.46 (br. t, J = 7.5) | 2.84 (m, J = 14) | 7.49 (q, J = 7) | 8.45 (br. d, J = 12) | 7.96 (br. d, J = 11) | 2.85 (m) |
| H–C(17) | 2.89 (dd, J = 5, 2.2) | 5.43 (m, J = 11, 8, 2) | 2.03 (d, J = 7) | 6.54 (tq, J = 11, 2) | 6.59 (m, J = 11.5, 15) | 2.38 (m, J = 7.5) | 5.38 (m, J = 14, 10.8) | 2.02 (d) | 6.54 (ddq, J = 12, 11, 1) | 6.59 (ddq, J = 11, 15, 1) | 5.4 (m, J = 11) |
| H–C(18) | 3.13 (m, J = 2.2, 5) | 6.06 (dq, J = 11.7) | – | 6.10 (dq, J = 10.5, 7) | 6.39 (m, J = 15, 7) | 1.65 (m, J = 7.5) | 5.74 (m, J = 10.8, 6.8) | – | 6.10 (dq, J = 11, 7) | 6.38 (dq, J = 15, 7) | 5.7 (m, J = 11) |
| CH₃(19) | 1.44 (d, J = 5) | 1.89 (dd, J = 7, 2) | – | 2.19 | 2.00 (br. d, J = 7) | 1.08 (t, J = 7.5) | 1.65 (d, J = 6.8) | – | 2.21 (dd, J = 7, 1) | 1.99 (br. d, J = 7) | 1.62 (d) |
| H–C(2') | 3.57 (m, J = 8, 6) | 3.57 (dq, J = 8.7, 6.3) | 3.58 (dq, J = 8, 6) | 3.55 (dq, J = 8.5, 6) | 3.57 (dq, J = 8.5, 6) | 3.56 (dq, J = 8.5, 6) | 3.56 (dq, J = 8, 6) | 3.54 (m, J = 8) | | | |
| H–C(3') | 3.21 (t, J = 9) | 3.21 (t, J = 9) | 3.20 (t, J = 9) | 3.21 (t, J = 9) | 3.22 (t, J = 9) | 3.23 (t, J = 9) | 3.23 (t, J = 8.5) | 3.35 (t, J = 9) | | | |
| H–C(4') | c) | 2.89 (ddd, J = 11.5, 9.3, 3) | 2.85 (m) | 2.88 (br. t, J = 10) | 2.92 (m) | 2.93 (m) | 2.9 (m) | 2.89 (m) | | | |
| H–C(5') | c) | 1.32 (q, J = 11.5) | c) | 1.30 (m, J = 11) | c) | 1.33 (m) | 1.33 (br. q, J = 11.5) | c) | | | |
| | c) | ca. 2.25 | c) | c) | c) | c) | 2.28 (m, J = 12) | c) | | | |

Table (cont.)

| | Hedamycin (1) | Rubiflavin A (4) | Rubiflavin B (5) | Rubiflavin C-1 (6) | Rubiflavin C-2 (7) | Rubiflavin D (8) | Rubiflavin E (9) | Rubiflavin F (10) |
|---|---|---|---|---|---|---|---|---|
| H–C(6') | 5.46 (br. d, $J = 9$) | 5.45 (m, $J = 10$) | 5.45 (br. d, $J = 9.5$) | 5.43 (br. d, $J = 9$) | 5.45 (br. d, $J = 9$) | 5.45 (br. d, $J = 9$) | 5.44 (br. d, $J = 9$) | 5.45 (br. d, $J = 9$) |
| $CH_3$(7') | 1.43 (d, $J = 7$) | 1.43 (d, $J = 6.3$) | 1.42 (d, $J = 6$) | 1.42 (d, $J = 6$) | 1.42 (d, $J = 6$) | 1.43 (d, $J = 6$) | 1.42 (d, $J = 6$) | 1.38 (d, $J = 6$) |
| $(CH_3)_2$N–C(4') | 2.32 (s)[d] | 2.33 (s)[d] | 2.33 (s)[d] | 2.31 (s)[d] | 2.35 (s)[d] | 2.36 (s)[d] | 2.35 (s)[d] | 2.38 (s)[d] |
| H–C(2'') | 4.07 (m, $J = 6$) | 4.05 (br. q, $J = 6.3$) | 4.05 (br. q, $J = 6.5$) | 4.04 (br. q, $J = 6.5$) | 4.08 (br. q, $J = 6.5$) | 4.07 (br. q, $J = 6.5$) | 4.04 (br. q, $J = 6.5$) | 3.85 (br. q, $J = 7$) |
| H–C(3'') | 3.36 (br. s)[c] | 3.34 (br. s)[c] | 3.33 (br. s)[c] | 3.32 (br. s)[c] | 3.36 (br. s)[c] | 3.35 (br. s)[c] | 3.35 (br. s)[c] | 3.30 (br. s)[c] |
| H–C(5'') | [c] | ca. 2.25 | [c] | [c] | [c] | [c] | 2.28 (m, $J = 12$) | [c] |
| | [c] | 2.60 (dd, $J = 14, 3$) | [c] | 2.59 (dd, $J = 13, 3$) | 2.58 (br. d, $J = 13$) | 2.59 (br. d, $J = 13$) | 2.60 (br. d, $J = 14.5$) | [c] |
| H–C(6'') | 5.46 (br. d, $J = 9$) | 5.45 (m, $J = 10$) | 5.45 (br. d, $J = 9.5$) | 5.43 (br. d, $J = 9$) | 5.45 (br. d, $J = 9$) | 5.45 (br. d, $J = 9$) | 5.44 (br. d, $J = 9$) | 4.91 (br. d, $J = 10.5$) |
| $CH_3$(7'') | 1.50 (d, $J = 6$) | 1.51 (d, $J = 6.3$) | 1.50 (d, $J = 6.5$) | 1.51 (d, $J = 6.5$) | 1.51 (d, $J = 6.5$) | 1.51 (d, $J = 6.5$) | 1.50 (d, $J = 6.5$) | 1.49 (d, $J = 7$) |
| $CH_3$(8'') | 0.71 (s) | 0.69 (s) | 0.69 (s) | 0.67 (s) | 0.69 (s) | 0.69 (s) | 0.71 (s) | 1.21 (s) |
| $(CH_3)_2$N–C(4'') | 2.20 (s)[d] | 2.22 (s)[d] | 2.25 (s)[d] | 2.18 (s)[d] | 2.21 (s)[d] | 2.23 (s)[d] | 2.20 (s)[d] | 2.22 (s)[d] |

[a])   Chemical shifts in ppm downfield from internal TMS; coupling constants in Hz; solvent $CDCl_3$.
[b])   Data from [18].
[c])   Chemical shift not determined due to signal overlap or folding.
[d])   May be interchanged.

fragment and a $CH_3CH_2$ group. Extensive decoupling experiments showed that these two fragments had to be linked by an additional $CH_2$ group. Its resonance was hidden under signals of the 2 amino sugar moieties but could be revealed at 2.38 ppm when the vinyl proton was irradiated and *Massiot's Fourier* transform difference-spectra method was used [16]. The configuration of the double bond was determined by NOE-difference spectroscopy: when $CH_3(15)$ was irradiated, an NOE was observed for $CH_2(17)$ and $H-C(3)$. Thus, rubiflavin D has a $(E)$-1-methyl-1-pentenyl side chain.

According to the $^1$H-NMR spectrum, the side chain of rubiflavin E (**9**) contained 2 vinyl protons (5.74 and 5.38 ppm), a $CH_2$ group (2.84 ppm), and 2 $CH_3$ groups (1.70 and 1.65 ppm). The resonances of 1 of the vinyl protons and of the $CH_2$ group overlapped with other signals, but their exact positions and splitting patterns could clearly be revealed by the difference-spectra method *(cf. Fig. 3)*. The spectral data indicated that the side chain in
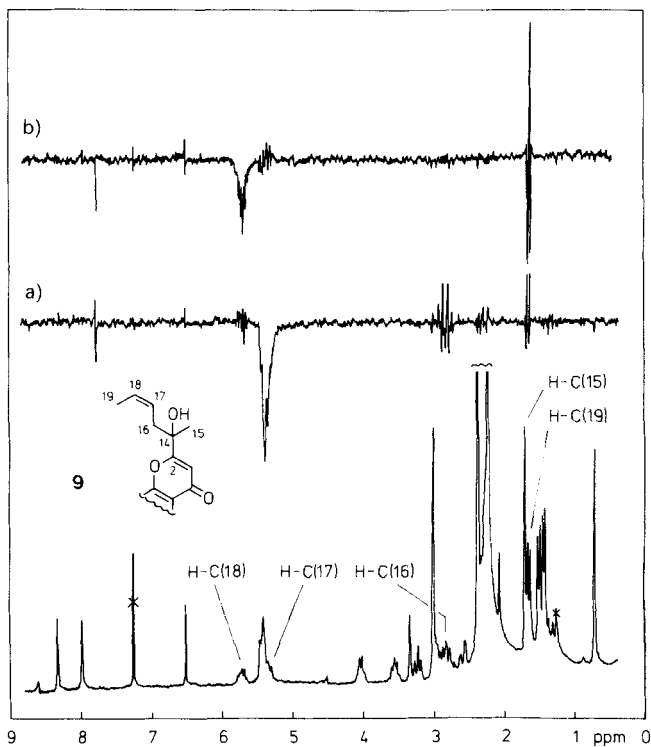


Fig. 3. *200-MHz-$^1$H-NMR spectrum of rubiflavin E* (**9**). a) Difference spectrum with irradiation of $H-C(17)$; b) difference spectrum with irradiation of $H-C(18)$.

this substance had to have the 1-methyl-3-pentenyl structure with $(Z)$-configuration $(J(17,18) = 10.8$ Hz) and with an additional function at C(1). The chemical shifts of the 1-methyl and of the $CH_2$ group of the side chain suggested that this additional functional group was an OH group. Thus, the side chain of **9** seemed to be the same that was found in $\beta$-indomycinone (**13**) [17]. The unpublished NMR data of **13** [18] were indeed in good agreement with those of rubiflavin E (**9**; *cf. Table*). It was, however, not possible to determine the configuration at C(14) relative to those in the amino-sugar rings E and F.

Two features in the $^1$H-NMR spectrum of rubiflavin F (**10**) were remarkable: first, the compound obviously contained the same 1-methyl-1-propenyl side chain as rubiflavin B ( = kidamycin; **5**), since the resonances of a vinyl proton at 7.49 ppm and of 2 $CH_3$ resonances at 2.02 and 2.00 ppm were observed. On the other hand, differences in the pattern of the sugar resonances with respect to rubiflavin B (**5**) could be seen. The signals of the ring-F protons, particularly those of $H-C(6'')$ and of $CH_3-C(4'')$, pointed to a changed configuration of this sugar

ring. A spectral and chromatographical comparison of rubiflavin F and its triacetate with isokidamycin (10; the C(6″) epimer that *Furukawa* obtained by acid treatment of kidamycin (5) [8]) and the corresponding triacetate revealed that rubiflavin F was indeed identical with isokidamycin (10).

The ¹H-NMR spectra of the two unpolar substances isolated from *Fraction 1*, the rubiflavinones C-1 (2) and C-2 (3), readily indicated that these compounds lacked the 2 amino-sugar moieties. Instead, 2 additional protons were attached to ring D and, together with H–C(9), gave rise to the same *ABC* pattern as the protons in 1,8-dihydroxyanthraquinone. The remaining resonances of the 4*H*-anthra[1,2-*b*]pyran-4,7,12-trione skeleton and of the side chains corresponded exactly to those of the rubiflavins C-1(6) and C-2(7). Therefore, 2 and 3 are the 'aglycones' of these rubiflavins. They have the same constitution as α-indomycinone (12) described many years ago by *Brockmann* [14]; the configuration of that latter compound had, however, never been determined [18]. The configuration of the trisubstituted double bond in the two rubiflavinones 2 and 3 was determined to be (*E*) by NOE-difference spectroscopy: when CH₃(15) was irradiated, an NOE could be observed for H–C(17) and H–C(3). Furthermore, irradiation of H–C(3) led to an NOE for CH₃(15), indicating that the conformation along the C(2)–C(14) bond was such that H–C(3) and CH₃–C(14) were synperiplanar.

**Discussion.** – Clearly, 'rubiflavin' is a mixture of several pluramycin-like antibiotics. Therefore, any biological experiments that were carried out with this mixture should be interpreted with caution. Furthermore, discussions of 'rubiflavin'-DNA and -protein interactions (*cf. e.g.* [5]) should be revised as far as molecular ratios are concerned, since 'rubiflavin' has now been shown to have roughly the double molecular weight than was thought earlier.

We were surprised to find two rubiflavinones (2 and 3) in the mixture. *Aszalos'* isolation and purification procedure included a step where the hydrochlorides were formed, separated, and then decomposed with Na₂CO₃ to give again the parent antibiotics [3]. Here, the rubiflavinones lacking the amino sugars should actually have been eliminated.

Rubiflavin F (10), which was shown to be identical with isokidamycin, might be an artifact formed during this step of the isolation procedure involving acid treatment. In fact, isokidamycin was prepared by *Furukawa* [8] by heating kidamycin (5) with *p*-toluenesulfonic acid in refluxing CHCl₃.

Rubiflavin C-2 (7) as well as rubiflavinone C-2 (3) might also be artifacts. We observed that rubiflavinone C-1 (2), when left in CDCl₃ solution for some days, slowly isomerized into 3. Thus the (*Z*)-double bond between C(17) and C(18) was converted into the more stable (*E*)-form. This isomerization was not observed – nor was it looked for – with rubiflavin C-1 (6), but it seems reasonable to assume that the same reaction will also take place with this compound. The fact that the terminal double bonds in rubiflavin A (4) as well as in rubiflavin E (9) have the (*Z*)-configuration might be considered as biogenetic evidence that rubiflavin C-1 (2) is the genuine antibiotic and rubiflavin C-2 (3) an artifact.

The absolute configurations of the compounds described here were not determined. But since all the rubiflavins are obviously biogenetically related and since kidamycin was found among them, it seem to be fair to assume that the absolute configurations of the rubiflavins are the same as those found for kidamycin (5) [8] and hedamycin (1) [19].

## Experimental Part

**General.** – Solvents: MeCN *puriss. (Fluka)*, NH₃ soln. *p.a. (Merck)*, techn. CH₂Cl₂ redistilled (*Chem. Fabrik, Schweizerhalle*), abs. MeOH *(Baker)*, *PIC* reagents *B-7* and *B-5* ( = heptane- and pentanesulfonic acid, resp., *Waters*). Solvents were removed *in vacuo* at 30–40° using a rotary evaporator. TLC: *Merck* precoated silica-gel plates (type *G60-F254*); CHCl₃/Et₃N 4:1 ($R_f$(I)) and toluene/Et₃N 4:1 ($R_f$(II)). HPLC: *Spectra-Physics* pump and gradient mixer *SP 8700*, variable-wavelength detector *SP 8400* set to 254 nm, *Hewlett-Packard* integrator *HP 3390A*; columns: silica gel, 4.6 × 250 mm, *LiChrosorb Si 60*, 7 µm *(Knauer)*; reversed phase, 4 × 250 mm, *Nucleosil 5 C8*, 5 µm *(Macherey & Nagel)*, 3.9 × 300 mm, *µ-Bondapak C 18*, 10 µm *(Waters)*, 8 × 250 mm, *LiChrosorb RP-18*, 7 µm *(Knauer)*. The samples used for spectroscopical identification were directly collected from HPLC. UV/VIS spectra: *Beckmann* mod. *25* spectrometer; due to the very small amounts of material on hand, exact molecular-extinction coefficients could not be determined; the absorbance relative to the strongest band is given. IR spectra: *Beckmann IR-8*. NMR spectra: *Bruker WH 90* (Institut für organische Chemie der Universität Basel; *K. Aegerter*), *Bruker WP 200 Sy* (Pharmazeutisches Institut der Universität Basel), *Bruker WM 250* (*Ciba-Geigy AG*, Basel; Dr. *G. Rist* and *S. Osswald*), *Bruker WH 360* (*Sandoz AG*, Basel; *H.-R. Loosli* and *L. Oberer*), *Bruker WH 400* (*Spectrospin AG*, Fällanden; *J. Sonderegger*). Mass spectra: *VG-70-250* mass spectrometer; substances for FAB-MS (Xe, 8 kV) were dissolved in a small amount of chlorobenzene, and this soln. was added to 3-nitrobenzyl alcohol which served as the matrix substance.

**Separation Procedure.** – *Prepurification.* Crude 'rubiflavin' (75 mg) was dissolved in 20 ml of CH₂Cl₂ and placed on top of a 30 g *Sephadex LH 20* column. CH₂Cl₂ (400 ml) and 0–25% MeOH/CH₂Cl₂ (400 ml) were used for elution. Fractions of 35 ml each were collected. The 2nd and 3rd fractions contained the interesting components (45.7 mg, 61%), $R_f$(I) 0.7–0.5. The subsequent fractions held 29 mg (39%) of polar material, $R_f$(I) 0.0–0.1, which was not further analyzed.

*First Fractionation by HPLC on Silica Gel.* The prepurified material (45.7 mg) was dissolved in 5 ml of CH₂Cl₂ and chromatographed on the *LiChrosorb Si 60* column (50 injections of 100 µl each) with CH₂Cl₂/MeOH/aq. NH₃ 930:65:4.5. Five fractions were collected; the solvent was removed immediately. *Fraction 1*, $k'$ 0–1.4, 7 mg; *Fraction 2*, $k'$ 1.4–3.9, 5.5 mg; *Fraction 3*, $k'$ 3.9–6.3, 15.7 mg; *Fraction 4*, $k'$ 6.3–7.1, 2.3 mg; *Fraction 5*, $k'$ 7.1–12.5, 11.4 mg.

*Isolation of Pure Compounds. Rubiflavinones C-1* (**2**) *and C-2* (**3**). Fraction 1 (7 mg) was rechromatographed on silica gel using CH₂Cl₂/MeOH/aq. NH₃ 980:20:2. Four single peaks were collected; peaks 2–4 were not further analyzed due to very low amounts of material. The 1st peak eluted ($k'$ 0.1) was re-chromatographed on the same column using CH₂Cl₂. A fraction at $k'$ 1.5 contained **2** and **3** as a 8:2 mixture (0.3 mg). Mixture **2/3** was separated by chromatography on *LiChrosorb RP-18* with MeOH: $k'$ 6.78 for **2** and $k'$ 5.64 for **3**.

*Rubiflavins A* (**4**) *and B* (**5**). Fraction 3 (18.8 mg) in 1.2 ml of MeOH was injected onto the *Nucleosil 5 C8* column in 48 portions of 25 µl each; MeOH/H₂O 3:1 containing one flask of *PIC B-7* reagent per liter was used as solvent. The 3 main components were collected and the solvent immediately removed. Then each of the 3 residues was dissolved in 30 ml of 5% aq. NaHCO₃ soln. and extracted with 4 × 30 ml of CHCl₃. The combined org. layers were washed with 100 ml of H₂O, dried (Na₂SO₄), and evaporated: **4**, $k'$ 2.4, 4.1 mg; **5**, $k'$ 3.3, 4.6 mg; rubiflavin C (mainly **6/7/8**), $k'$ 5.9, 3.6 mg. All components still contained 1 to 1.5 equiv. of heptanesulfonic acid *(PIC B-7)*; therefore, they were re-chromatographed on silica gel with CH₂Cl₂/MeOH/aq. NH₃ 970:30:4. This led to nearly pure **4** (1.7 mg) and **5** (2.8 mg).

*Rubiflavins C-1* (**6**), *C-2* (**7**), *and D* (**8**). Rubiflavin C (mainly **6/7/8**; 2.2 mg) was dissolved in 0.3 ml of MeOH and rechromatographed on the *µ-Bondapak C 18* column with H₂O/MeCN 3:2 containing one flask of *PIC B-5* reagent per liter as solvent. The 3 main components were collected and the solvent removed at once. Then, each of the residues was dissolved in 20 ml of 5% aq. NaHCO₃ soln. and extracted with 4 × 25 ml of CHCl₃. The org. layers were combined, washed with 50 ml of H₂O, dried (Na₂SO₄), and evaporated: **6**, 0.8 mg; **7**, 0.6 mg; **8**, 0.3 mg. Again all these compounds still contained 1–1.5 equiv. of pentanesulfonic acid *(PIC B-5)*, which was removed by chromatography on silica gel with CH₂Cl₂/MeOH/aq. NH₃ 970:30:4, giving 0.9 mg of pure **6**, 0.5 mg of pure **7**, and 0.6 mg of pure **8** from 1.6, 1.1, and 1.2 mg of the respective prepurified substances.

*Rubiflavin E* (**9**). Fraction 4 proved to be a pure compound.

*Rubiflavin F* (**10**). Fraction 5 (11.4 mg) in 1 ml of CH₂Cl₂ was re-chromatographed (10 injections of 100 µl each) on silica gel using CH₂Cl₂/MeOH/aq. NH₃ 950:50:4.5. Besides the main peak of rubiflavin F (**10**, 1.3 mg; $k'$ 0.66, contained some impurity), small amounts of 5 other components were obtained, but were not investigated further.

**Data of the Rubiflavins A–F (4–10) and of the Rubiflavinones C-1 and C-2 (2 and 3, resp.).** – *11-Hydroxy-5-methyl-2-[(1 E,3 Z)-1-methyl-1,3-pentadienyl]-4*H-*anthra[1,2-*b*]pyran-4,7,12-trione* ( = *Rubiflavinone C-1;* **2**). Yellow solid, $R_f$ 0.36 (silica gel, CHCl$_3$). UV (EtOH): 203 (0.75), 228 (1.00), 240 (sh, 0.89), 288 (0.62), 308 (sh, 0.48), 402 (0.27). $^1$H-NMR (250 MHz, CDCl$_3$): *Table.* MS (EI, 70 eV): 386 (57, $M^{+\cdot}$), 371 (100), 357 (9), 343 (20), 281 (53), 252 (5), 197 (6), 139 (6), 133 (6), 105 (6), 91 (6).

*11-Hydroxy-5-methyl-2-[(1 E,3 E)-1-methyl-1,3-pentadienyl]-4*H-*anthra[1,2-*b*]pyran-4,7,12-trione* ( = *Rubiflavinone C-2;* **3**). Yellow solid, $R_f$ 0.35 (silica gel, CHCl$_3$). UV (EtOH): 204 (0.54), 230 (1.00), 240 (sh, 0.90), 289 (0.67), 307 (sh, 0.54), 400 (0.29). $^1$H-NMR (250 MHz, CDCl$_3$): *Table.* EI-MS (70 eV): 386 (63, $M^{+\cdot}$), 371 (100), 357 (8), 343 (20), 281 (64), 252 (6), 197 (5), 193 (6), 168 (6), 139 (9), 133 (10), 105 (9), 91 (12), 77 (7), 65 (5).

*11-Hydroxy-5-methyl-2-[2-methyl-3-((Z)-1-propenyl)oxiran-2-yl]-8-[2,3,6-trideoxy-3-(dimethylamino)-β-*D-arabino-*hexopyranosyl]-10-[2,3,6-trideoxy-3-(dimethylamino)-3-*C-methyl-α-L-lyxo-*hexopyranosyl]-4*H-*anthra[1,2-*b*]pyran-4,7,12-trione* ( = *Rubiflavin A;* **4**). Orange solid, $R_f$ (I) 0.66, $R_f$ (II) 0.33. UV (EtOH): 203 (0.79), 245 (1.00), 265 (sh, 0.64), 293 (sh, 0.28), 435 (0.18). $^1$H-NMR (360 MHz, CDCl$_3$): *Table.* $^{13}$C-NMR (90.6 MHz, CDCl$_3$)$^3$): 188.1 (C(12)); 183.3 (C(7)); 179.0 (C(4)); 167.5 (C(2)); 159.9 (C(11)); 156.2 (C(12b)); 149.8 (C(5)); 140.1 (C(8)); 138.5 (C(10)); 137.4 (C(6a)); 134.0 (C(18)); 133.1 (C(9)); 126.4, 125.9 (C(4a), C(7a), C(6)); 123.3 (C(17)); 119.2 (C(12a)); 116.2 (C(11a)); 110.0 (C(3)); 77.3 (C(2')); 75.2 (C(6')); 71.9 (C(3')); 70.9 (C(3'')); 69.7 (C(6'')); 67.4 (C(4')); 67.2 (C(2'')); 61.6 (C(16)); 59.1 (C(14)); 57.3 (C(4'')); 40.4 ((CH$_3$)$_2$N–C(4')); 36.8 ((CH$_3$)$_2$N–C(4'')); 33.6 (C(5'')); 28.4 (C(5')); 24.1 (C(13)); 18.9 (C(7')); 17.7 (C(7'')); 14.5 (C(15)); 13.8 (C(19)); 12.3 (C(8'')). FAB-MS: 731 ([$M$ + H]$^+$).

*11,3',3''-Tri-O-acetylrubiflavin A.* To a soln. of A **4** (2.1 mg, 2.8 μmol) in 22 μl of pyridine, 12.5 μl of Ac$_2$O (0.13 mmol) were added. The soln. was warmed to 40° for 5 min and then kept at r. t. in the dark for 72 h. After addition of 15 ml of 10% aq. KHCO$_3$ soln., the mixture was extracted with 4 × 25 ml of CHCl$_3$, the combined org. layer was washed with 50 ml of H$_2$O, dried (Na$_2$SO$_4$), and evaporated: 2.3 mg (93%) of triacetate. Light-orange solid, $R_f$ 0.35 (silica gel, CHCl$_3$/MeOH 9:1). $^1$H-NMR (90 MHz, CDCl$_3$)$^3$): 8.32 (*s*, H–C(9)); 7.86 (*s*, H–C(6)); 6.45 (*s*, H–C(3)); 6.03 (*m*, *J* = 11, 7, H–C(18)); 5.47 (*m*, H–C(6'), H–C(6'')); 5.4 (*m*, H–C(17)); 5.23 (*d*, *J* = 4.5, H–C(3'')); 4.91 (*t*, *J* = 9.5, H–C(3')); 4.34 (*m*, *J* = 6.5, 4.5, H–C(2'')); 3.98 (*d*, *J* = 7.5, H–C(16)); 3.67 (*m*, *J* = 9, 6, H–C(2')); 2.97 (*s*, CH$_3$(13)); 2.45 (*s*, AcO–C(11)); 2.32 (*s*, (CH$_3$)$_2$N); 2.27 (*s*, (CH$_3$)$_2$N); 2.18 (*s*, AcO–C(3'')); 2.14 (*s*, AcO–C(3')); 1.87 (*dd*, *J* = 7, 1.5, CH$_3$(19)); 1.77 (*s*, CH$_3$(15)); 1.42 (*d*, *J* = 6.5, CH$_3$(7'')); 1.31 (*d*, *J* = 6, CH$_3$(7')); 0.97 (*s*, CH$_3$(8'')).

*11-Hydroxy-5-methyl-2-[(E)-1-methyl-1-propenyl]-8-[2,3,6-trideoxy-3-(dimethylamino)-β-*D-arabino-*hexopyranosyl]-10-[2,3,6-trideoxy-3-(dimethylamino)-3-*C-methyl-α-L-lyxo-*hexopyranosyl]-4*H-*anthra[1,2-*b*]pyran-4,7,12-trione* ( = *Rubiflavin B;* **5**). Orange solid, $R_f$ (I) 0.66, $R_f$ (II) 0.29. $[\alpha]_D^{23}$ = +440 ± 40° (*c* = 0.015, CH$_2$Cl$_2$) ([8]: +476° (*c* = 0.986, CHCl$_3$)). UV (EtOH): 215 (0.76), 244 (1.00), 269 (0.69), 433 (0.20). $^1$H-NMR (90 MHz, CDCl$_3$): *Table.* FAB-MS: 689 ([$M$ + H]$^+$).

*11,3',3''-Tri-O-acetylrubiflavin B.* To a soln. of **5** (2.0 mg, 2.9 μmol) in 23 μl of pyridine, 12 μl of Ac$_2$O (0.12 mmol) were added. The soln. was warmed to 40° for 5 min and then kept in the dark at r.t. for 70 h. After addition of 20 ml of 10% aq. KHCO$_3$ soln., the mixture was extracted with 4 × 25 ml of CHCl$_3$, the combined org. layer washed with 60 ml of H$_2$O, dried, and evaporated to yield 2.2 mg (93%) of triacetate. Bright-orange solid, $R_f$ 0.36 (silica gel, CHCl$_3$/MeOH 9:1). $^1$H-NMR (90 MHz, CDCl$_3$)$^3$): 8.32 (*s*, H–C(9)); 7.82 (*s*, H–C(6)); 7.45 (*m*, H–C(16)); 6.35 (*s*, H–C(3)); 5.43 (*m*, *J* = 10, H–C(6'), H–C(6'')); 5.24 (*d*, *J* = 4.5, H–C(3'')); 4.91 (*t*, *J* = 9.5, H–C(3')); 4.34 (*m*, *J* = 6.5, 4.5, H–C(2'')); 3.67 (*m*, *J* = 9, 6, H–C(2')); 2.98 (*s*, CH$_3$(13)); 2.51 (*s*, AcO–C(11)); 2.32 (*s*, (CH$_3$)$_2$N); 2.27 (*s*, (CH$_3$)$_2$N); 2.19 (*s*, AcO–C(3'')); 2.14 (*s*, AcO–C(3')); 1.99 (*m*, CH$_3$(15), CH$_3$(17)); 1.43 (*d*, *J* = 6.5, CH$_3$(7'')); 1.31 (*d*, *J* = 6, CH$_3$(7')); 0.97 (*s*, CH$_3$(8'')).

*11-Hydroxy-5-methyl-2-[(1 E,3 Z)-1-methyl-1,3-pentadienyl]-8-[2,3,6-trideoxy-3-(dimethylamino)-β-*D-arabino-*hexopyranosyl]-10-[2,3,6-trideoxy-3-(dimethylamino)-3-*C-methyl-α-L-lyxo-*hexopyranosyl]-4*H-*anthra[1,2-*b*]pyran-4,7,12-trione* ( = *Rubiflavin C-1;* **6**). Orange solid, $R_f$ (I) 0.67, $R_f$ (II) 0.27. UV (EtOH): 204 (0.73), 230 (0.93), 245 (1.00), 280 (sh, 0.59), 313 (sh, 0.40), 408 (0.23), 429 (0.23). $^1$H-NMR (200 MHz, CDCl$_3$): *Table.* FAB-MS: 715 ([$M$ + H]$^+$).

*11-Hydroxy-5-methyl-2-[(1 E,3 E)-1-methyl-1,3-pentadienyl]-8-[2,3,6-trideoxy-3-(dimethylamino)-β-*D-arabino-*hexopyranosyl]-10-[2,3,6-trideoxy-3-(dimethylamino)-3-*C-methyl-α-L-lyxo-*hexopyranosyl]-4*H-*anthra[1,2-*b*]pyran-4,7,12-trione* ( = *Rubiflavin C-2;* **7**). Orange solid, $R_f$ (I) 0.67, $R_f$ (II) 0.27. UV (EtOH): 203 (0.69), 230 (0.98), 245 (1.00), 281 (sh, 0.63), 311 (sh, 0.45), 400 (0.23), 431 (0.23). $^1$H-NMR (200 MHz, CDCl$_3$): *Table.* FAB-MS: 715 ([$M$ + H]$^+$).

*11-Hydroxy-5-methyl-2-[( E)-1-methyl-1-pentenyl]-8-[2,3,6-trideoxy-3-(dimethylamino)-β-D-arabino-hexo-pyranosyl]-10-[2,3,6-trideoxy-3-(dimethylamino)-3-C-methyl-α-L-lyxo-hexopyranosyl]-4H-anthra[1,2-b]pyran-4,7,12-trione* ( = *Rubiflavin D;* **8**). Orange solid, $R_f$ (I) 0.68, $R_f$ (II) 0.33. UV (EtOH): 203 (0.71), 214 (0.79), 244 (1.00), 268 (sh, 0.75), 434 (0.18). $^1$H-NMR (200 MHz, CDCl$_3$): *Table.* FAB-MS: 717 ([$M$ + H]$^+$).

*11-Hydroxy-5-methyl-2-[( Z)-1-hydroxy-1-methyl-3-pentenyl]-8-[2,3,6-trideoxy-3-(dimethylamino)-β-D-ara-bino-hexopyranosyl]-10-[2,3,6-trideoxy-3-(dimethylamino)-3-C-methyl-α-L-lyxo-hexopyranosyl]-4H-anthra[1,2-b]pyran-4,7,12-trione* ( = *Rubiflavin E;* **9**). Orange solid, $R_f$ (I) 0.50, $R_f$ (II) 0.14. UV (EtOH): 204 (0.68), 244 (1.00), 265 (sh, 0.58), 292 (sh, 0.26), 434 (0.18). $^1$H-NMR (200 MHz, CDCl$_3$): *Table.* FAB-MS: 733 ([$M$ + H]$^+$).

*11-Hydroxy-5-methyl-2-[( E)-1-methyl-1-propenyl]-8-[2,3,6-trideoxy-3-(dimethylamino)-β-D-arabino-hexo-pyranosyl]-10-[2,3,6-trideoxy-3-(dimethylamino)-3-C-methyl-β-L-lyxo-hexopyranosyl]-4H-anthra[1,2-b]pyran-4,7,12-trione* ( = *Rubiflavin F;* **10**). Orange solid, $R_f$ (I) 0.60, $R_f$ (II) 0.20. UV (EtOH): 213 (0.73), 243 (1.00), 269 (0.68), 432 (0.20). $^1$H-NMR (90 MHz, CDCl$_3$): *Table.* FAB-MS: 689 ([$M$ + H]$^+$).

*11,3′,3″-Tri-O-acetylrubiflavin F.* A soln. of **10** (1.2 mg, 1.7 µmol) in 20 µl of pyridine and 5 µl of Ac$_2$O (60 µmol) was warmed to 40° for 5 min and kept in the dark at r.t. for 72 h. Then, 10 ml of 10% aq. KHCO$_3$ were added and the mixture extracted with 3 × 25 ml of CHCl$_3$. The combined org. layer was washed with 30 ml of H$_2$O, dried (Na$_2$SO$_4$), and evaporated: 0.8 mg (56%) of triacetate. Yellow solid, $R_f$ 0.14 (silica gel, CHCl$_3$/MeOH 9:1). $^1$H-NMR (90 MHz, CDCl$_3$)$^3$): 8.34 (*s*, H–C(9)); 7.83 (*s*, H–C(6)); 7.38 (*m*, H–C(16)); 6.35 (*s*, H–C(3)); 5.38 (br. *d*, *J* = 9, H–C(6′)); 4.93 (br. *s*, H–C(3″)); 4.9 (*m*, H–C(6″)); 4.86 (*t*, *J* = 9, H–C(3′)); 3.94 (br. *q*, *J* = 6.5, H–C(2″)); 3.70 (*m*, H–C(2′)); 2.98 (*s*, CH$_3$(13)); 2.50 (*s*, AcO–C(11)); 2.33 (*s*, (CH$_3$)$_3$N); 2.26 (*s*, AcO–C(3″)); 2.17 (*s*, (CH$_3$)$_2$N); 2.14 (*s*, AcO–C(3′)); 1.99 (*s*, CH$_3$(15)); 1.99 (CH$_3$(17)); 1,27 (*d, J* = 6, CH$_3$(7″)); 1.20 (*d, J* = 5.5, CH$_3$(7′)); 1.20 (*s*, CH$_3$(8″)).

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