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ABSOLUTE AND RELATIVE CONFIGURATION OF ERYTHROSKYRIN

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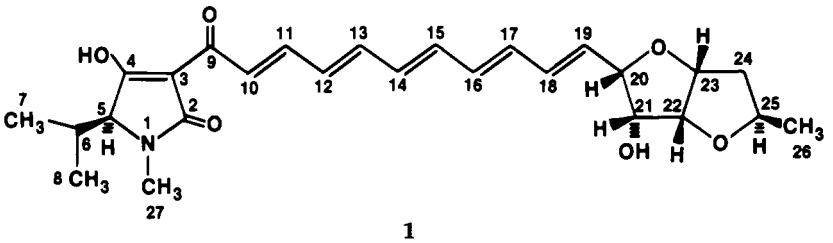
ABSTRACT.—We have reisolated erythroskyrin [**1**] from *Penicillium islandicum* and have determined the relative stereochemistry of the compound through extensive ^{1}H- and ^{13}C-nmr studies. The absolute stereochemistry was determined by nmr studies of the *O*-methylmandelate esters.

The gross structure of erythroskyrin [**1**] was elucidated in 1964 (1,2); however, the stereochemistry of five of the six chiral centers was not addressed. In view of the unique structure of this mycotoxin and in connection with the biological evaluation of erythroskyrin by the National Cancer Institute, we have carried out nmr and cd studies with the aim of establishing the relative and absolute stereochemistry of the chiral centers. We also report details of the reisolation procedure because the strains of *Penicillium islandicum* in which erythroskyrin occurs as the sole major pigment (1,3) are not currently available, and the isolation from strain NRRL 1036 involves separation from numerous anthraquinone pigments. We have also found that several of the original ^{1}H-nmr assignments were incorrect and have assigned these and the carbon resonances.



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RESULTS AND DISCUSSION

PROTON ASSIGNMENTS.—The 300 MHz ^{1}H nmr is tabulated in Table 1. It is essentially identical to that published by Shoji *et al.* (1), with allowances for the higher field strength. A COSY spectrum (4) and specific decoupling experiments involving irradiation of resonances at 2.29, 5.76, 3.83, 4.54, 4.80, and 4.21 ppm established correct assignments unambiguously. It is seen that the assignments by Shoji *et al.* for protons at positions 6, 20, 21, 22, 23, and 24 were in error.

Of particular interest is the assignment of a minor singlet that occurs at 2.95 ppm, just downfield from the *N*-methyl singlet. Shoji *et al.* assigned this to 21-OH, since it disappeared with acid addition. We interpret this signal as representative of the *N*-methyl singlet of a minor conformer in the sample and believe that the signal disappearance with acid is due to a shift under the *N*-methyl singlet. Spectrum doubling has been reported before for tetronomycin (5), and the presence of other minor signals seen best in the ^{13}C-nmr spectrum and in the HETCOR experiment (6) supports this interpretation.

NOe connectivities (7) are reported in Table 1. They confirm the COSY results and indicate further that the fused tetrahydropyran ring system protons 19–23 must all

TABLE 1. ^{1}H-nmr Spectrum of Erythroskyrin (300 MHz, $CDCl_3$).

Proton	Chemical shift	*J* (Hz)	NOe to proton no.
5	3.55 d	3.0	6, 7, 8, 27
6	2.29 m	3.0, 7.0, 7.1	5, 7, 8, 27
7	1.08 d (3H)	7.0	5, 6, 8, 27
8	0.94 d (3H)	7.1	5, 6, 7, 27
10	7.17 dd	15.1	11, (12–18)
11	7.48 dd	15.1, 11.3	10, (12–18)
12–18	6.3–6.7 m	—	10, 11, 19, 20
19	5.76 dd	14.7, 6.5	(12–18), 20
20	4.06 dd	6.5, 8.0	(12–18), 19, 21
21	3.83 dd	8.0, 5.2	20, 22
22	4.54 dd	5.0, 5.2	21, 23
23	4.80 dd	4.9, 5.0	22, 24a
24a	1.68 ddd	10.6, 13.6, 5.0	23, 24b
24b	2.24 dd	5.0, 13.6	24a
25	4.21 ddq	5.0, 6.0, 10.6	26
26	1.31 d (3H)	6.0	25, 24a
27 (*N*-Me)	3.00 s (3H)	—	5, 6, 7, 8

exist in *cisoid* relative configurations (nOe connectivities are not expected for *transoid* systems). The observed scalar couplings are consistent with such a system.

This left the relative configuration at C-25 unassigned. Though we did not see a NOESY signal between H-25 and either proton H-24a or H-24b, we were able to observe a weak difference nOe between the resonances at 4.21 ppm and 2.24 ppm (H-24b). Irradiation of the methyl group (1.30 ppm) gave a difference nOe to H-25 only. These results established the relative stereochemistry at C-25 as shown. Scalar couplings in this system were compared to those of literature model compounds, but no clear deductions could be drawn by this method.

CARBON ASSIGNMENTS.—The HETCOR experiment and APT (8) spectra allowed the ^{13}C-nmr assignments to be made for all aliphatic carbons with attached protons, except for the two isopropyl methyls (separated by 0.02 ppm). The unsaturated resonances 12–18 were not well resolved in the proton spectrum. This left a pair of singlets at 171.95 and 173.66 (carbons 2 and 9) and a pair of doublets at 82.43 and 82.41 (carbons 22 and 23) unassigned.

The singlets were assigned by INAPT experiments (9). Thus, irradiation of the proton signal at 3.55 ppm (H-5) gave carbon signals at 17.16, 71.14, 173.66, and 194.03 ppm, corresponding to carbons 8, 5, 2, and 4, respectively. Irradiation of the *N*-methyl signal at 3.00 ppm produced carbon signals at 71.14 and 173.66 ppm. C-9 was assigned as 171.95 by its INAPT signals on irradiation of 7.17 and 7.48 ppm, respectively. Additional assignments into the unsaturated system were gained by further INAPT work. Irradiation of 7.17 ppm, for example, showed that 131.35 belonged to C-12. The ^{13}C-nmr spectrum of erythroskyrin is tabulated in Table 2.

CIRCULAR DICHROISM.—The *p*-bromobenzoate of erythroskyrin (at position 21 as verified by its bathochromic uv shift with acid) was examined (10). The coupling between the polyene and the *p*-bromobenzoate chromophores was too weak to give measurable cd absorption. Similarly, reductive ozonolysis of erythroskyrin (11,12) led to the diol, which was then di-*p*-bromobenzoylated. The cd absorption of this ester was also too weak to measure. This result is consistent with the dihedral angle between chromophores being near 0°.

MOSHER-TROST CHIRAL ESTER METHOD.—Both mandelate esters of erythrosky-

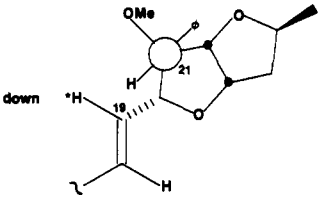
TABLE 2. ^{13}C-nmr Spectrum of Erythroskyrin (50 MHz, $CDCl_3$).

Carbon	Chemical shift	Proton correlation
2	173.66 s	—
3	100.82 s	—
4	194.03 s	—
5	71.14 d	3.55
6	29.00 d	2.29
7	17.17 q[a]	0.94, 1.08
8	17.15 q[a]	0.94, 1.08
9	171.95 s	—
10	121.16 d	7.17
11	143.87 d	7.48
12	131.35 d	(obscured)
13–16	132.64, 133.02, 138.07, 142.59 (all d)	6.3–6.7
17	135.09 d	(obscured)
18	132.22 d	(obscured)
19	132.93 d	5.76
20	84.23 d	4.06
21	77.96 d	3.83
22	82.43 d[b]	4.54
23	82.41 d[b]	4.80
24	43.55 t	1.68, 2.24
25	77.30 d	4.21
26	20.62 q	1.31
27	27.38 q	3.00

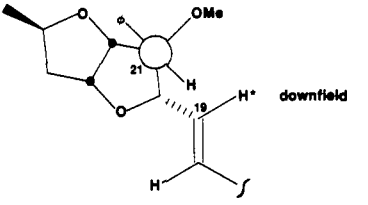
[a,b]Assignment may be reversed.

rin were prepared by the method of Trost *et al.* (13). This enabled assignment of C-21 as *R*. Coupled with the relative assignments established by nOe, this permitted the complete assignment of this portion of the molecule to be made.

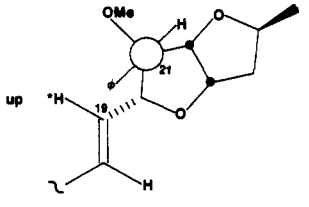
ABSOLUTE CONFIGURATION OF ERYTHROSKYRIN.—The absolute configuration



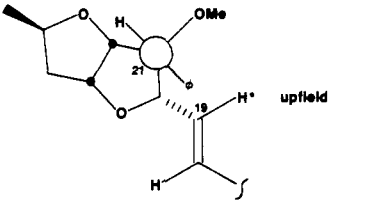
S-ester and C-21–(*S*)
*predicts 19-H downfield to *R*-ester



R-ester and C-21(*R*)
*predicts 19-H downfield compared to *S*-ester



R-ester and C-21–(*S*)
*predicts 19-H upfield to *S*-ester



S-ester and C-21(*R*)
*predicts 19-H upfield compared to *R*-ester

FIGURE 1. Stereochemistry of *O*-methyl mandelate esters of erythroskyrin.

of the tetramic acid portion of the molecule has previously been determined as 5*S* by ozonolysis to L(+)-*N*-methylvaline (2). The absolute configuration of erythroskyrin must be 5*S*, 20*S*, 21*R*, 22*R*, 23*R*, 25*R*. It is highly likely that the 5*S* center exists in an equilibrium mediated via a protonated resonance form.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—All operations were performed under yellow fluorescent lights to avoid light-induced degradation of erythroskyrin. All chromatographic fractions were immediately evaporated because erythroskyrin has been found to degrade in MeOH solution at 3° in the dark. Nmr spectra were acquired on a Nicolet NT-300WB and a Varian XL-200 spectrometer in $CDCl_3$. All ^{1}H-nmr chemical shifts were referenced to internal TMS, and all ^{13}C-nmr chemical shifts were referenced against $CDCl_3$ (77.0 ppm). The 2D nOe experiment (NOESY) used a delay of 4 sec, a 90-degree pulse of 8 μsec, a mixing time of 700 msec, a 512 block size, 128 increments, quadrature detection in both dimensions, zero filling once in t_1, acquisitions a multiple of 16. The long-range coupling experiment INAPT used the pulse sequence of Bax *et al.* (9). A decoupler field strength $\gamma H_2 = 25$ Hz was used to generate a selective 90 degree proton pulse of 10 msec. The polarization transfer delays d_1 and d_2 were optimized for $J_{CH} = 6$ Hz. Circular dichroic measurements were obtained on a JASCO J-40 spectropolarimeter in EtOH. All solvents were hplc grade, and $CHCl_3$ was hydrocarbon-stabilized.

ERYTHROSKYRIN ISOLATION.—*P. islandicum* NRRL 1036 was grown in stationary culture in the absence of light on Czapek-Dox liquid medium, 32 × 1 liters. The mycelium was filtered, freeze-dried, and defatted with hexane. Threefold Me_2CO extraction yielded 63.7 g of crude extract. Elution via flash chromatography on Si gel (four 7 × 16 cm columns) in $CDCl_3$-HOAc, 100:0.1 (v/v), then $CDCl_3$-MeOH-HOAc, 100:1:0.1 (v/v), then MeOH, yielded purified erythroskyrin in the 1% MeOH fractions. The evaporated residue (7.9 g) was rechromatographed on a single flash silica column (7 × 16 cm) using $CDCl_3$-butyl acetate-HOAc, 50:50:0.1 (v/v), followed by $CDCl_3$-MeOH-HOAc, 100:1:0.1 (v/v), then MeOH, yielding a pure sample of erythroskyrin (0.76 g) and a mixed fraction (6.0 g) which was rechromatographed in $CDCl_3$-MeOH-HOAc, 100:1:0.1 (v/v) to yield a further 2.0 g of pure erythroskyrin. This material gave nmr, uv, and ir spectra which matched published values (1). An authentic standard of erythroskyrin was not available. High resolution fabms from glycerol/DMF (negative ion mode) gave a molecular ion $[M-H]^-$ of *m/z* 454.22296 (calcd for $C_{26}H_{32}N_1O_6$, 454.22293).

MONO-*p*-BROMOBENZOATE OF ERYTHROSKYRIN.—The bromobenzoate was prepared from erythroskyrin in pyridine at 50° using an excess of *p*-bromobenzoyl chloride and isolated by flash chromatography. Ms, uv, and nmr spectra were consistent with a single ester substitution at position 21.

REDUCTIVE OZONOLYSIS OF ERYTHROSKYRIN.—Treatment of erythroskyrin (5 mg, 1×10^{-5} mol) with ozone in MeOH at −78° for 3 h followed by ethanolic $NaBH_4$ (excess) for 1 h at −78° and warming to 25°, addition of H_2O, washing with $CHCl_3$ (3 × 10 ml), and removal of solvent produced the diol. Pyridine, DMAP (catalyst), and *p*-bromobenzoylchloride were added to the diol and stirred at 65° for 12 h to give the dibenzoate (14) after work-up with hexane-EtOAc (5:1 v/v). The cd spectrum showed none of the exciton coupling expected, probably because the dihedral angle between the *p*-bromobenzoate chromophores is close to 0°.

CHIRAL *O*-METHYLMANDELATE ESTER PREPARATION.—Esterification of erythroskyrin with *R* and *S* *O*-methylmandelic acid used the following procedure (13,15): A solution of the carboxylic acid (2×10^{-5} mol), DCC (2×10^{-5} mol), erythroskyrin (9.7 mg, 2×10^{-5} mol), and DMAP (one crystal) in CH_2Cl_2 (1 ml) stood at 25° for 4 h. The *N,N*-dicyclohexyl urea formed was filtered off, and the filtrate was washed with H_2O (3 × 1 ml), 5% HOAc (3 × 1 ml), and H_2O again (3 × 1 ml), dried ($MgSO_4$), and subjected to preparative tlc over silica (toluene-Me_2CO, 1:1 v/v). This material (ca. 100 μg of each ester) was used for determination of absolute stereochemistry by the Mosher-Trost method (Figure 1). Both esters gave ^{1}H-nmr spectra consistent with the formation of a single ester at C-21. The *S*-mandelate ester showed a 19-H resonance in the ^{1}H nmr at 5.50 ppm. The corresponding *R*-mandelate ester showed 19-H at 5.70 ppm. Eims of the two esters were superimposable, showing fragments at 224 (15%), 143 (15), 121 (10), 99 (25), 83 (15), 69 (15), 54 (100), and 41 (30).

SODIUM SALT OF ERYTHROSKYRIN.—The sodium salt was prepared by brief reaction of erythroskyrin with one equivalent of NaOH in MeOH solution, evaporation to dryness, dissolution in distilled H_2O at 0.2 mg/ml, filtration, and lyophilization. The ^{1}H nmr of this material was essentially identical to that of free erythroskyrin. Fabms showed an $[M+Na]^+$ peak at *m/z* 478 as expected. A uv maximum of 390 nm (log $\epsilon = 4.32$) was consistent with base-shifted erythroskyrin. Conversion back to free erythroskyrin with

acid treatment confirmed that the skeleton was intact. The bioactivity of the salt prepared in this way was superior to free erythroskyrin in several biological models due to increased H_2O solubility.

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