Notes

METABOLIC PRODUCTS OF MICROORGANISMS. 260†

NAPHTHGERANINES, NEW NAPHTHOQUINONE ANTIBIOTICS FROM *Streptomyces* sp.

PETER WESSELS, AXEL GÖHRT and AXEL ZEECK*

Institut für Organische Chemie, Universität Göttingen, Tammannstr. 2, D-3400 Göttingen, FRG

HANNELORE DRAUTZ and HANS ZÄHNER

Institut für Biologie II, Universität Tübingen, Auf der Morgenstelle 28, D-7400 Tübingen, FRG

(Received for publication March 4, 1991)

In the course of our chemical screening program we detected a new naphthoquinone complex containing the naphthgeranines and the naphtherythrines[2] produced by *Streptomyces* sp. (strain Tü 3556). This paper deals with the purification, structural elucidation and biological properties of the naphthgeranines A to E, which are similar to the recently described naphterpin[3].

After isolation from a soil sample collected in Nepal, the strain Tü 3556 was cultivated in 500 ml flasks of 10 liter fermenters at 27°C for 96 hours or 120 hours using mannitol 2% and soybean meal 2% as a culture medium. Metabolites from the culture filtrate were adsorbed on Amberlite XAD-16 before being eluted with methanol from the resin. The mycelium was extracted with methanol. The combined solutions were concentrated under reduced pressure and the aqueous residues extracted with ethyl acetate. The isolation of the naphthgeranines from the resulting raw product was successful by using a silica gel column (flash chromatography) developed with a chloroform - methanol gradient of increasing polarity: Naphthgeranines A (95 : 5), B (9 : 1), E (8 : 2), C and D (7 : 3). Further purification steps were carried out using silica gel (naphthgeranines A and B: chloroform - methanol (95 : 5 and 9 : 1, respectively), naphthgeranine C: chloroform - methanol - acetic acid (95 : 5 : 0.5)) and Sephadex LH-20 (chloroform - methanol (1 : 1)) yielding the antibiotics as yellow to red amorphous substances. In various fermentations we obtained the naphthgeranines in quantities up to 9 (A); 2 (B); 3 (C); 1 (D) and <1 (E) mg/liter culture broth. The physicochemical properties of the antibiotics are shown in Table 1.

The shift of the adsorption band at 445 nm in the UV/VIS spectra to 510 nm by changing from neutral to basic medium pointed to a 5-hydroxy-1,4-naphthoquinone (juglone) as chromophore of naphthgeranine A (**1**)[4]. NMR investigations, especially 2D-NMR experiments ($^1J_{C,H}$ and $^nJ_{C,H}$ heteronuclear shift correlation: $^{13}C,^1H$-QUICK and correlation spectroscopy *via* long range coupling (COLOC), $^1H,^1H$-COSY) in combination with the mass spectra led to the partial structures **1a** and **1b** in Fig. 1. From the 4a-H,12b-H coupling constant (5 Hz) the *cis*-linkage of the cyclohexene ring A with the dihydropyran ring B could be deduced. The spectroscopical data, however, particulary those obtained from the COLOC-experiment, showed no correlation signals which allowed determination of the regiochemistry at the quinoid 6a,12a double bound. Information could be derived from the ^{13}C NMR carbonyl signals of C-7 (δ 182.5) and C-12 (δ 184.0) which were assigned by the $^3J_{C,H}$ correlation from δ 7.00 (11-H) to δ 184.0 (C-12). The signal of C-7 was expected downfield compared to that of C-12, because the former carbonyl group is strongly chelated by 8-OH[5]. The opposite result pointed to an alkoxy substituent at C-6a[6,7] leading to formula **1** for naphthgeranine A.

To confirm this result by an X-ray analysis we crystallized naphthgeranine A (**1**) by liquid-liquid diffusion from an acetone - water solvent system at 8°C. A small crystal with approximate dimensions $0.11 \times 0.38 \times 0.46$ mm was chosen for an X-ray work. The crystal data and intensity data were collected by graphite monochromated MoKα radiation. The crystal data are as follows: $C_{20}H_{20}O_5$, FW = 340.4. Monoclinic, space group $P2_1$. Cell dimensions, $a = 5.5470(10)$, $b = 14.322(3)$, $c = 10.170(2)$ Å, $\beta = 95.18°$ (2), $U = 804.6(3)$ Å^3. $Z = 2$, $D_{calc} = 1.405$ mg/m^3, μ for Mok$\alpha = 0.094$ mm^{-1}, Siemens-Stoe AED2 diffractometer, data collection with profile-fitting method[8] at -80°C, 2θ range = 8 to 50°, 2,952 reflections measured, 2,829 unique reflections, 2,671

† Part 259: See ref 1.

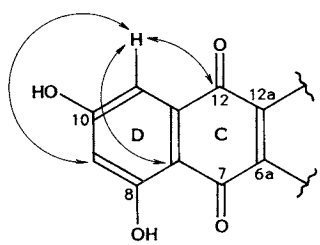
with $|F|>4\sigma_F$ treated as observed. The crystal structure was solved by direct methods based on SHELXS-86[9] and the atomic parameters were refined by the full matrix least squares methods. Although all H-atom positions were located by difference electron-density synthesis, a riding model with idealized hydrogen geometry was employed for refining them. The H-atom displacement parameters were refined isotropically. The anisotropic refinement converged at R=0.0331 (wR=0.0454 with

Table 1. Physico-chemical properties of the naphthgeranines A to E (**1**~**5**).

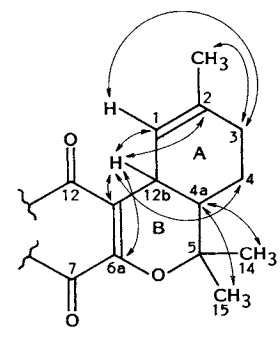
	1	**2**	**3**	**4**	**5**
Appearance	Orange needles	Orange powder	Orange powder	Orange powder	Darkred powder
MP (°C)	180 (dec)	165 (dec)	200 (dec)	210 (dec)	61
Molecular formula	$C_{20}H_{20}O_5$	$C_{20}H_{20}O_6$	$C_{20}H_{20}O_7$	$C_{20}H_{20}O_8$	$C_{20}H_{16}O_7$
FD-MS (M^+)	340	356	372	388	—
HREI-MS (M^+)[a]	340.1318	356.1260	372.1209	370.1053[b]	368.0896
$[\alpha]_D^{20}$	−317° (*c* 0.12, $CHCl_3$-MeOH, 1:1)	−361° (*c* 0.11, MeOH)	−309° (*c* 0.08, MeOH)	−304° (*c* 0.06, MeOH)	—
UV λ_{max}^{MeOH} nm (ε)	210 (21,480), 268 (20,000), 296 (11,710) 395 (2,640), 445 (2,590)	214 (17,880), 266 (10,350), 311 (9,480), 389 (2,640), 445 (2,720)	215 (19,910), 268 (10,910), 304 (9,960), 396 (2,810), 437 (2,760)	217 (18,540), 267 (12,890), 314 (10,530), 387 (3,250), 445 (2,630)	207 (25,550), 295 (15,190), 368 (2,700), 400 (2,700), 523 (2,700)
$\lambda_{max}^{MeOH+NaOH}$ nm (ε)	229 (22,030), 287 (18,110), 330 (6,410), 510 (3,350)	229 (20,600), 292 (12,380), 329 (5,540), 510 (3,100)	229 (23,250), 293 (14,080), 328 (7,960), 507 (4,620)	231 (20,160), 291 (15,840), 330 (9,560), 509 (5,230)	226 (20,640), 321 (19,360), 426 (4,750), 607 (3,920)
CD $\lambda_{extreme}^{MeOH}$ nm ($[\theta]^{23}\times10^{-3}$)	463 (−6), 392 (+1), 388 (+2), 296 (−17), 267 (−17), 233 (−21), 211 (+68)	461 (−6), 388 (+1), 357 (−1), 297 (−13), 268 (−17), 232 (−19), 210 (+54)	464 (−10), 380 (+2), 296 (−21), 265 (−22), 232 (−28), 209 (+58)	454 (−11), 373 (+3), 318 (−21), 264 (−24), 231 (−21), 209 (+54)	—
IR (KBr) cm^{-1}	3380, 1628, 1611, 1576	3430, 1635, 1594	3420, 1634, 1595	3400, 1635, 1595	3380, 3260 (sh), 1710, 1620, 1610, 1570
Rf value (TLC, silica gel)					
$CHCl_3$-MeOH (95:5)	0.61	0.26			
$CHCl_3$-MeOH (9:1)		0.48	0.25	0.09	0.35

[a] Found as calcd.
[b] $M^+ - H_2O$.

Fig. 1. $J_{C,H}$ Long range couplings leading to the naphthoquinone moiety (**1a**) and the terpenoide moiety (**1b**) of naphthgeranine A (**1**).



1a



1b

Fig. 2. Computer generated perspective drawing of naphthgeranine A (**1**).

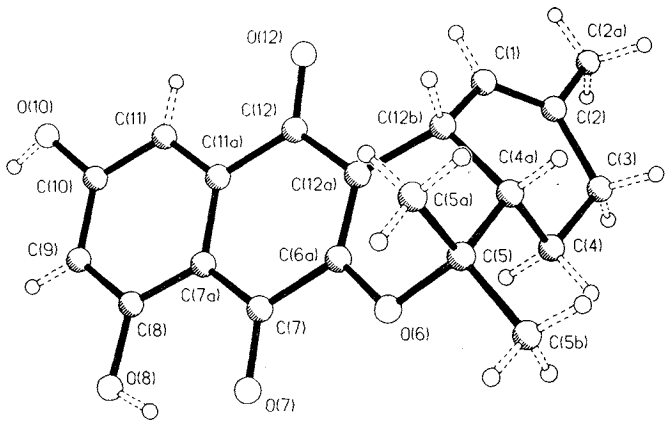


Table 2. ^{13}C NMR signals of the naphthgernines A to E (**1**~**5**).

Carbon	**1**[a]	**2**[b]	**3**[c]	**4**[d]	**5**[e]
1	119.7 d	122.7 d	126.0 d	126.0 d	129.4 d
2	135.7 s	138.6 s	138.6 s	138.2 s	120.8 s
3	29.3 t	25.4 t	64.0 d	69.1[f] d	152.7[f] s
4	20.1 t	19.9 t	28.5 t	70.4[f] d	110.6[g] d
4a	39.4 d	39.9 d	34.4 d	39.4 d	140.5 s
5	80.4 s	80.9 s	80.7 s	81.2 s	81.9 s
6a	152.8 s	153.7 s	154.1 s	155.7 s	158.7[f] s
7	182.5 s	182.6 s	182.3 s	184.0 s	183.7 s
7a	107.5 s	108.1 s	107.7 s	108.8 s	108.9 s
8	165.2[f] s	164.3[f] s	165.4[f] s	167.1[g] s	167.7[h] s
9	106.3 d	107.1 d	106.8 d	107.2 d	107.6 d
10	164.0[f] s	164.2[f] s	164.3[f] s	165.6[g] s	165.5[h] s
11	108.8 d	109.1 d	109.0 d	109.6 d	110.5[g] d
11a	134.5 s	134.4 s	134.4 s	136.3 s	136.1 s
12	184.0 s	183.9 s	184.1 s	184.5 s	184.1 s
12a	123.3 s	122.9 s	121.3 s	123.7 s	117.1 s
12b	30.8 d	30.8 d	30.9 d	30.2 d	128.5 s
13	23.1 q	66.8 t	65.6 t	65.0 t	60.9 t
14	24.5 q	24.9 q	24.7 q	25.9 q	27.6 q
15	25.1 q	25.7 q	25.4 q	27.0 q	27.6 q

[a] 50.3 MHz, in $CDCl_3$-CD_3OD; [b] 125.7 MHz in $CDCl_3$; [c] 125.7 MHz in $CDCl_3$-CD_3OD; [d] 50.3 MHz in CD_3OD; [e] 125.7 MHz in CD_3OD; [f~h] assignments may be interchanged.

δ values in ppm relative to internal TMS, multiplicity assignments by attached proton test (ATP).

weights $W^{-1}=\sigma^2_F+0.0005F^2$). Further details of the crystal structure investigations are available on request from the Fachinformationszentrum Energie, Physik, Mathematik GmbH, D-7514 Eggenstein-Leopoldshafen 2 (FRG), on quoting the depository number CSD-55230, the names of the authors and the journal citation. A computer generated perspective drawing of naphthgeranine A (**1**) is given in Fig. 2.

Naphthgeranine A (**1**) shows very close correspondence to the recently described naphterpin (**6**)[3] in which an additional methyl group appears at C-9. The absolute configuration of **6** has been established by an X-ray analysis of its bromoacetate making use of the anomalous scattering of the bromine atom. From comparison of the optical rotation values of **1** and **6** ($[\alpha]_D$ −648°, *c* 0.1, $CHCl_3$), it followed that the absolute configuration is ambiguous. Surprisingly, the optical rotation values differ more than it would be expected considering the small structural difference.

The structures of the naphthgeranines B to E (**2** to **5**) could be determined by comparison of the NMR and mass spectroscopical data with those of

Table 3. ^{1}H NMR signals (200 MHz) of the naphthgeranines A to E (**1**~**5**).

Proton	**1**[a]	**2**[a]	**3**[b]	**4**[b]	**5**[c]
1-H	6.04 dd (5, 1)	6.34 d (5)	6.57 d (6)	6.67 d (4)	8.43 s
3-H_{ax}	1.86~2.07 m	2.00~2.18 m	—	—	—
3-H_{eq}			4.21 dd (4, 2)	3.93 d (3)	—
4-H_{ax}	1.29 m	1.29 m	1.40 dt (4, 13)	—	6.82 s
4-H_{eq}	1.94 m	2.00 m	2.05 dt (13, 2)	4.27 dd (3, 2)	
4a-H	1.78 m	1.82 m	2.19 ddd (13, 6, 2)	2.21 dd (7, 2)	—
9-H	6.46 d (2)	6.46 d (2)	6.42 d (2)	6.41 d (2)	6.57 d (2)
11-H	7.00 d (2)	6.98 d (2)	6.95 d (2)	6.98 d (2)	7.16 d (2)
12b-H	3.47 t (5)	3.53 t (5)	3.56 t (6)	3.64 dd (7, 4)	—
13-H_2	1.68 s (3H)	3.97 d (13), 4.02 d (13)	4.06 d (13), 4.16 d (13)	4.12 s	4.82 s
14-H_3	1.33 s	1.35 s	1.36 s	1.40 s	1.69 s
15-H_3	1.55 s	1.57 s	1.53 s	1.67 s	1.69 s
8-OH	11.94[d] s	11.70[d] s	12.01[e] s	12.04[e] s	12.18 s

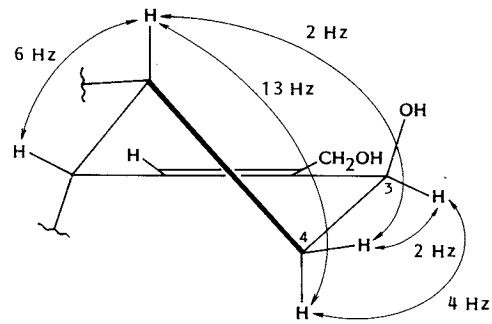
[a] $CDCl_3$-CD_3OD; [b] CD_3OD; [c] acetone-d_6; [d] $CDCl_3$.
δ values in ppm relative to internal TMS, J (Hz) in brackets.

1. In the case of naphthgeranine B (**2**) an additional oxygen atom was suggested from MS data (Table 1). The main differences in the NMR spectra between **1** and **2** were found for C-13 and the adjacent protons (Tables 2 and 3) indicating that the methyl group of **1** became hydroxylated. Thus naphthgeranine B (**2**) is a hydroxy derivative of **1**.

For naphthgeranine C (**3**), it was evident that a further hydroxy group is present compared with **2**. Its position at C-3 or C-4 became clear from an additional ^{13}C signal at δ 64.0, which was attributed to an oxygenated methine, and the loss of a methylene signal in the region of 20 to 30 ppm (Table 2). Analysis of the ^{1}H,^{1}H-coupling pattern of ring A supported by information obtained from an ^{1}H,^{1}H-COSY experiment led to the situation depicted in Fig. 3. Thus naphthgeranine C (**3**) is a 3,13-dihydroxy derivative of **1**. The values of the coupling constants of ring A unambiguously proved the (*S*)-configuration at C-3 assuming that the stereochemistry of the skeleton was unchanged compared with **1**.

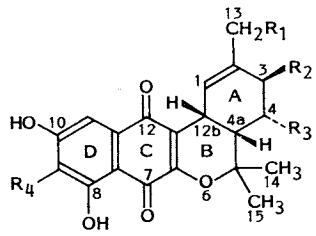
The analogous examination of the spectroscopical data of naphthgeranine D (**4**) led to the result that it was a 3,4,13-trihydroxy derivative of **1**. The last methylene group of ring A has changed to a hydroxylated methine group. A 2-Hz coupling constant between 4-H and 4a-H indicated the eq position of the former and therefore the (*R*)-configuration at C-4. The configuration at C-3 of **4** remained unsettled because the coupling constant between 3-H and 4-H did not allow distinction between an quasiaxial or quasiequatorial position. The relative configuration of 3-OH and 4-OH was

Fig. 3. Vicinal $J_{H,H}$ coupling constants of ring A in naphthgeranine C (**3**).

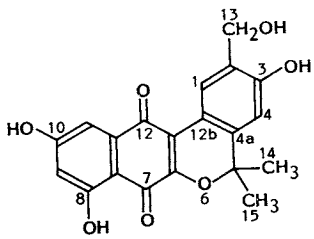


established *via* an oxidation of **4**[10,11]. We used the TLC method developed by CIFONELLI and SMITH[12] showing colorless spots on a dark blue colored adsorbent. Considering the yellow color of **4** we used aquayamycin (**7**)[13] as a positive standard. Compared with **7** undoubtedly no reaction took place with naphthgeranine D (**4**). Thus the hydroxy groups of ring A should have *trans* configuration.

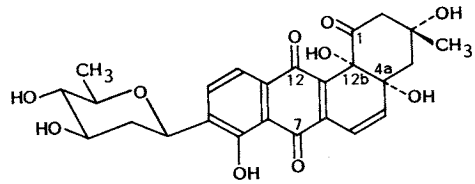
Naphthgeranine E (**5**) is distinguished from the other components by its dark red color which changed to blue in alkaline solutions. The ^{13}C NMR spectra in the region up to 50 ppm only showed one signal at δ 27.6 for the geminal methyl groups. The increase of signals in the aromatic/olefinic region compared with **1** led to the conclusion that ring A was aromatizated as shown in formula **5**. This was in agreement with the molecular formula $C_{20}H_{16}O_7$ (Table 1) indicating a higher degree of unsaturation and with the missing optical activity of naphthger-



	R_1	R_2	R_3	R_4
Naphthgeranine A (**1**)	H	H	H	H
Naphthgeranine B (**2**)	OH	H	H	H
Naphthgeranine C (**3**)	OH	OH	H	H
Naphthgeranine D (**4**)	OH	OH	OH	H
Naphterpin (**6**)	H	H	H	CH_3



Naphthgeranine E (**5**)



Aquayamycin (**7**)

anine E (**5**). Compared with **1** the additional singlets at δ 8.43 and 6.82 showed two aromatic protons in *para* position to each other. The possible precursor of **5** might be naphthgeranine D (**4**).

Naphterpin (**6**)[3] has been isolated as antioxidative agent. For **1** and **2** we detected a weak antibacterial and antifungal activity in the agar disc diffusion assay with MIC values between 0.1 to 1 mg/ml. The more polar **2** is less active. An anti-HIV-activity of **1**, **2** and **3** could not be observed up to a concentration of 50 μg/ml. At this concentration at toxic effect of the naphthgeranines against the MT4 leukemia cells used in the test[14] limited further investigations. The cytocidal activities against various tumor cell lines are summarized in Table 4. The naphthgeranines and naphterpin[3], respectively, belong to the small group of microbial quinones[15] in which structural elements derive from the polyketide pathway and from mevalonic acid

Table 4. Cytocidal activities of naphthgeranines A to C (**1**~**3**) against various tumor cell lines *in vitro* (IC_{50}: μg/ml)[17].

	Mouse leukemia L1210	Human leukemia HT29	Lung carcinoma A549
1	60	13	18
2	50	21	48
3	>10	>10	>10

building blocks as well[16]. These classes of secondary metabolites are more typical for plants and fungi than for *Streptomyces*.

Acknowledgment

This work was supported by the Fonds der Chemischen Industrie.

References

1) Decker, H.; H.-P. Fiedler, H. Zähner, H. Heitsch & W. A. König: Structure-activity relationship of the nikkomycins. J. Gen. Microbiol. 137: 1805~1813, 1991
2) Wessels, P.: Strukturaufklärung neuer Naphthochinon-Antibiotika aus *Streptomyces* sp. Tü 3556 und Beiträge zum chemischen Screening mit extrem thermophilen Archaebakterien. Ph. D. Thesis, Univ. Göttingen, 1990
3) Shin-ya, K.; S. Imai, K. Furihata, Y. Hayakawa, Y. Kato, G. D. Vanduyne, J. Clardy & H. Seto: Isolation and structural elucidation of an antioxidative agent, naphterpin. J. Antibiotics 43: 444~447, 1990
4) Singh, I.; R. T. Ogata, R. E. Moore, C. W. J. Chang & P. J. Scheuer: Electronic spectra of substituted naphthoquinones. Tetrahedron 24: 6053~6073, 1968
5) Bowden, B. F.; D. W. Cameron, M. J. Crossley, G. I. Feutrill, P. G. Griffiths & D. P. Kelly: Carbon-13 N.M.R. studies of 1,4-naphthoquinones and 9,10-anthraquinones. Aust. J. Chem. 32: 769~777, 1979
6) Höfle, G.: ^{13}C-NMR-Spektroskopie chinoider Verbindungen: II. Substituierte 1,4-Naphthochinone und Anthrachinone. Tetrahedron 33: 1963~1970, 1977
7) McDonald, I. A.; T. J. Simpson & A. F. Sierakowski: ^{13}C N.M.R. spectral studies of some naturally occurring quinones and related compounds. Aust. J. Chem. 30: 1727~1734, 1977
8) Clegg, W.: Faster data collection without loss of precision. An extension of the learnt profile method. Acta Crystallogr. (A) 37: 22~28, 1981
9) Sheldrick, G. M.: SHELXS-86. University of Göttingen, 1986
10) Buist, G. J.; C. A. Bunton & W. C. P. Hipperson:

The mechanism of oxidation of α-glycols by periodic acid. Part X. The oxidation of pinacol, and a general discussion of stability of periodate esters and their role in the mechanism of oxidation. J. Chem. Soc. (B) 1971: 2128~2142, 1971

11) ANGYAL, S. J. & R. J. YOUNG: Glycol fission in rigid systems. II. The cholestane-3β,6,7-triols. Existence of a cyclic intermediate. J. Am. Chem. Soc. 81: 5251~5255, 1959

12) CIFONELLI, J. A. & F. SMITH: Detection of glycosides and other carbohydrate compounds on paper chromatograms. Anal. Chem. 26: 1132~1134, 1954

13) OHTA, K.; E. MIZUTA, H. OKAZAKI & T. KISHI: The absolute configuration of P-1894B, a potent prolyl hydroxylase inhibitor. Chem. Pharm. Bull. 32: 4350~4359, 1984

14) JURKIEWICZ, E.; P. PANSE, K.-D. JENTSCH, H. HARTMANN & G. HUNSMANN: *In vitro* anti-HIV-1 activity of chondroitin polysulphate. AIDS 3: 423~427, 1989

15) HENKEL, T. & A. ZEECK: Secondary metabolites by chemical screening. 15. Structure and absolute configuration of naphthomevalin, a new dihydronaphthoquinone antibiotic from *Streptomyces* sp. J. Antibiotics 44: 665~669, 1991

16) SHIN-YA, K.; K. FURIHATA, Y. HAYAKAWA & H. SETO: Biosynthetic studies of naphterpin, a terpenoid metabolite of *Streptomyces*. Tetrahedron Lett. 31: 6025~6026, 1990

17) CARMICHAEL, J.; W. G. DEGRAFF, A. F. GAZDAR, J. D. MINNA & J. B. MITCHELL: Evaluation of a tetrazolium-based semiautomated colorimetric assay: Assessment of chemosensitivity testing. Cancer Res. 47: 936~942, 1987