CHEMICAL AND BIOLOGICAL STUDIES ON CALVATIC ACID AND ITS ANALOGS

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An interesting antibiotic with a diazene *N*-oxide structure, calvatic acid (**1a**), was isolated by UMEZAWA's group from cultures of *Calvatia craniformis* (SHW.) Fr.[1], by ANKE from a strain of *Licoperdon pyriforme*† and by ourselves from cultures of *Calvatia lilacina* (BERK.) Henn. P.[2]. The first group has also reported a total synthesis of the antibiotic in four steps starting from *p*-hydrazinobenzoic acid hydrochloride.

Our continuing interest in the chemical and biological properties of diazene *N*-oxide derivatives[3], has led us to develop a new synthetic route to **1a**. We used 4-nitrosobenzoic acid (**2a**) as starting material. This compound can be easily obtained by the photo-isomerisation of 4-nitrobenzaldehyde dissolved in distilled water[4]. A pyridine solution of **2a**, treated at room temp with stoichiometric quantities of cyanamide and (diacetoxyiodo)benzene, gives good yields of **1a**. In the same way we prepared 2-(cyano-*N*,*N*,*O*-azoxy)benzoic acid (**1b**) starting from 2-nitrosobenzoic acid (**2b**) obtained by photo-isomerization of 2-nitrobenzaldehyde dissolved in benzene[5].

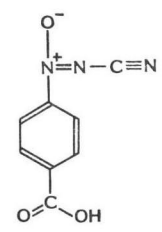
This synthesis is suitable for the preparation of ONN-azoxy-cyanides in which the azoxy function is linked to various carriers[3].

To gain more insight into the structure-activity relationships of **1a**, we prepared and studied for their antimicrobial activities its analogs 4-(phenylsulfonyl-*N*,*N*,*O*-azoxy)benzoic acid (**3**), 4-(ethoxycarbonyl-*N*,*N*,*O*-azoxy)benzoic acid (**4**), 4-(carbamoyl-*N*,*N*,*O*-azoxy)benzoic acid (**5a**) and 2-(carbamoyl-*N*,*N*,*O*-azoxy)benzoic acid (**5b**). The synthetic routes for obtaining these compounds are given Scheme 1.

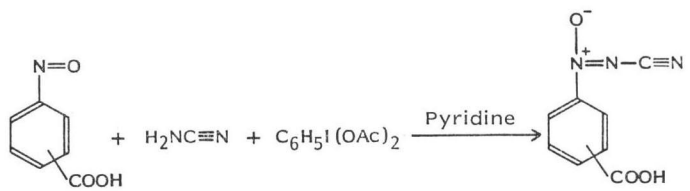
All the compounds are characterized, as is **1a**, by the presence on the benzene ring both of a carboxylic group and an azoxy function containing an electron withdrawing group. They have been screened *in vitro* for their antimicrobial activity against Gram-positive and Gram-negative bacteria and against fungi (see Table 1).

The compounds **1b**, **3**, **4**, **5a** do not show any relevant activity against the tested fungi, as is the case with the reference antibiotic, and they are also generally inactive against Gram-negative bacteria, with the only partial exception of **1b**. The two *ortho*-derivatives **1b** and **5b** (inactive) are less active than their *para*-isomers **1a** and **5a**.

We have also undertaken a study into the antimitotic properties of the *para*-derivatives **1a**, **3**,



1a



2a = 4-COOH
2b = 2-COOH

1a = 4-COOH
1b = 2-COOH

† ANKE, T.: Institute of Biology, Tubinga Univ., private communication.

Table 1. Antibacterial and antifungal spectra of the reference antibiotic **1a** and its analogs.

Compounds	MICs (μg/ml)*											
	S.a. 209P	*S.a.* 153	*M.l.*	*B.s.*	*M.s.*	*E.c.*	*Sa.a.*	*P.a.*	*K.p.*	*C.a.*	*T.m.* 20	*T.m.* (DeCarls)
1a	1.55	1.55	0.39	3.12	6.25	6.25	1.55	6.25	50	>100	>100	>100
1b	50	50	50	100	6.25	50	25	>100	50	>100	>100	>100
3	6.25	12.5	50	>100	>100	>100	>100	>100	>100	>100	>100	>100
4	6.25	6.25	>100	50	>100	>100	>100	>100	>100	>100	>100	>100
5a	50	25	12.5	50	>100	>100	50	100	>100	>100	>100	>100
5b	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100

Abbreviation: *S.a.* 209P; *Staphylococcus aureus* 209P, *S.a.* 153; *Staphylococcus aureus* 153, *M.l.*; *Micrococcus luteus* ATCC 9341, *B.s.*; *Bacillus subtilis* ATCC 6633, *M.s.*; *Mycobacterium smegmatis* ATCC 607, *E.c.*; *Escherichia coli* B, *Sa.a*; *Salmonella abortivoequina* ATCC 9842, *P.a.*; *Pseudomonas aeruginosa* F.I., *K.p.*; *Klebsiella pneumoniae* ATCC 10031, *C.a.*; *Candida albicans* F.I., *T.m.* 20; *Trichophyton mentagrophytes* 20, *T.m.* (DeCarls); *Trichophyton mentagrophytes* (DeCarls).

* Minimum inhibitory concentrations (MICs) were determined by the agar dilution method following previously established procedures[6].

Scheme 1.

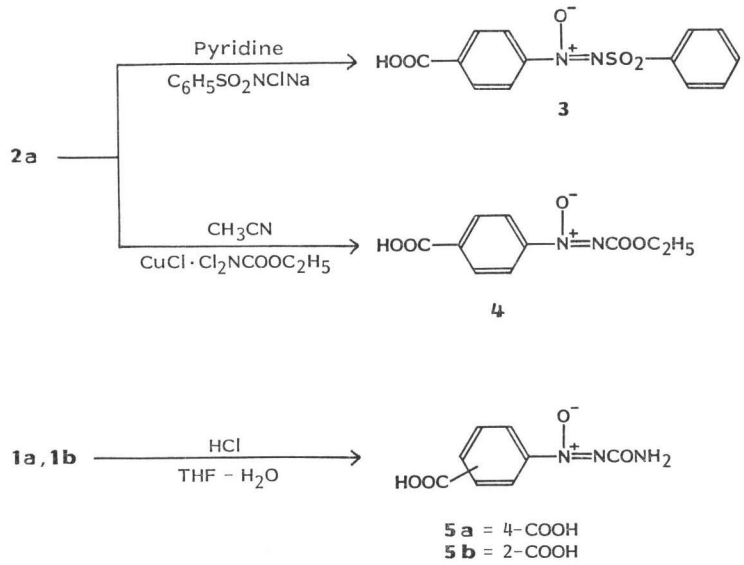


Table 2. Percentage inhibition of [^{3}H]colchicine binding to rat liver soluble tubulin after 30 minutes of incubation with calvatic acid (**1a**) and its *para*-analogs[a].

Compound[b]	Inhibition (%) Concentrations of compound (mmol)			
	0.5	1	2.5	5
1a	49 (2)[c]	66 (2)	85 (1)	96 (1)
3	66 (3)	90 (2)	97 (1)	99 (1)
4	36 (2)	99 (2)	99 (1)	99 (2)
5a	0 (3)	0 (2)	44 (1)	84 (1)

[a] The ability of the compounds to inhibit the binding between [^{3}H]colchicine and tubulin was determined according to the procedure previously reported[7].

[b] **1a**, **3**, **4** were tested as sodium salts.

[c] The number of experiments is reported in round brackets.

4, and **5a**, using a tubulin binding assay as a pre-screen. All the tested compounds are able to inhibit the binding of [^{3}H]colchicine to rat liver tubulin. The results are reported in Table 2. Details on this part of the work will be published elsewhere.

Experimental

Melting points were observed on a capillary Buchi 512 apparatus and are uncorrected. IR spectra, taken on a Perkin-Elmer 781 spectrophotometer, and ^{1}H NMR spectra, recorded in DMSO-d_6 solution (TMS reference) on a Jeol GX 270/89 instrument, are in keeping with the proposed structures.

2a and **2b** were prepared according to the literature methods[4,5] with the modifications reported below.

2-Nitrosobenzoic Acid (**2b**)

A solution of 2-nitrobenzaldehyde (2.00 g) in 150 ml benzene was irradiated over 6 hours using the full mercury arc of an immersion medium pressure lamp (Applied Photophysics Ltd.). The precipitate was collected by filtration and crystallized from alcohol (yield 50%): MP 205°C (dec) (ref[5] 205~210°C (dec)).

Anal Calcd for $C_7H_5NO_3$:
C 55.63, H 3.34, N 9.27.
Found: C 55.56, H 3.38, N 9.19.

4-Nitrosobenzoic Acid (**2a**)

A solution of 4-nitrobenzaldehyde (1.50 g) in 2 liters of lukewarm water was irradiated over 6 hours using the above reported lamp. The precipitate was collected by filtration, dried and washed with $CHCl_3$ and then with Me_2CO (yield 80%): MP 250°C (dec) (ref[4] 260°C (dec)).

Anal Calcd for $C_7H_5NO_3$:
C 55.63, H 3.34, N 9.27.
Found: C 55.91, H 3.51, N 9.23.

4-(Cyano-*N*,*N*,*O*-azoxy)benzoic Acid (Calvatic Acid) (**1a**)

A stirred solution of **2a** (0.604 g, 4.00 mmol) and cyanamide (0.202 g, 4.80 mmol) in 16 ml dry pyridine was treated at 30°C with (diacetoxyiodo)-

benzene (1.546 g, 4.80 mmol) in portions over 15 minutes. The reaction solution was stirred continuously for 1 hour at 30°C and then poured dropwise into 150 ml stirred 7% HCl. The precipitate was collected by filtration, washed with H_2O, dried and purified by flash chromatography (Silica gel Merck Kieselgel 60, 230~400 mesh ASTM; eluent $CHCl_3$ containing EtOAc 0~70%). The product was dissolved in Me_2CO in the presence of animal carbon. The solution obtained after filtration of the carbon, added dropwise with H_2O, separated **1a** (yield 77%): MP 203~204°C (dec) (ref[2] 198~199°C (dec), ref[1] 182~183°C (dec)). The IR spectrum of the product was identical to that of the antibiotic isolated from cultures of *C. lilacina*.

Anal Calcd for $C_8H_5N_3O_3$:
C 50.27, H 2.64, N 21.98.
Found: C 50.04, H 2.45, N 21.64.

2-(Cyano-*N*,*N*,*O*-azoxy)benzoic Acid (**1b**)

This compound was prepared starting from **2b** in a similar way to that of **1a**. The mixture obtaining after treating the reaction solution with 7% HCl 200 ml, was extracted with EtOAc. The organic layers, dried on $MgSO_4$, were evaporated under vacuum. The residue was dissolved in Me_2CO containing animal carbon. The solution obtained after filtration of the carbon, added dropwise with H_2O, precipitated **1b** (yield 50%): MP 159~160°C (dec); IR ν_{max}^{KBr} cm^{-1} 2220 (C≡N), 1460, 1330 (N(O)=N).

Anal Calcd for $C_8H_5N_3O_3$:
C 50.27, H 2.64, N 21.98.
Found: C 50.20, H 2.46, N 21.60.

4-(Phenylsulfonyl-*N*,*N*,*O*-azoxy)benzoic Acid (**3**)

To a stirred solution of **2a** (0.604 g, 4.00 mmol) in 12 ml dry pyridine, chloramine-B (1.068 g, 5.00 mmol) was added in portions over 20 minutes. The reaction mixture was stirred continuously for one night at room temp and then was heated over 2 hours at 80°C, finally it was poured dropwise into stirred and ice-water cooled 7% HCl 200 ml. The precipitate was collected by filtration, dried and dissolved in alcohol containing animal carbon. The solution obtained after filtration of the carbon, added dropwise with H_2O precipitated **3** (yield 75%): MP 228°C (dec); IR ν_{max}^{KBr} cm^{-1} 1470, 1350 (N(O)=N),1290, 1170 (SO_2).

Anal Calcd for $C_{13}H_{10}N_2O_5S$:
C 50.93, H 3.29, N 9.15.
Found: C 51.05, H 3.18, N 9.05.

4-(Ethoxycarbonyl-*N*,*N*,*O*-azoxy)benzoic Acid (**4**)

To an ice-water cooled and stirred suspension of **2a** (0.604 g, 4.00 mmol) in 20 ml dry acetonitrile, *N*,*N*-dichlorourethane (0.630 g, 4.37 mmol) and CuCl (0.792 g, 8.00 mmol) were slowly added. The reaction mixture was stirred at room temp over 18 hours. After this time it was filtered and the solid collected was washed with acetonitrile. The filtered solution and the acetonitrile used for washing were combined and evaporated under vacuum and the residue dissolved in alcohol containing animal carbon. The solution recovered after filtration of the carbon, added dropwise with H_2O, precipitated **4** (yield 52%): MP 198~199°C (dec); IR ν_{max}^{KBr} cm^{-1} 1750, 1485, 1340 (N(O)=N), 1235 (COO-C_2H_5).

Anal Calcd for $C_{10}H_{10}N_2O_5$:
C 50.42, H 4.23, N 11.76.
Found: C 50.40, H 4.05, N 11.63.

4-(Carbamoyl-*N*,*N*,*O*-azoxy)benzoic Acid (**5a**)

Into a 0°C cooled solution of **1a** (0.200 g, 1.04 mmol) in 2 ml of a mixture THF - H_2O (6 : 1), hydrogen chloride gas was bubbled over 10 minutes. The precipitate was collected by suction. The mother liquor added with H_2O provided another portion of the product. The combined crops were dried (yield 95%) and quantitatively crystallized from EtOH - H_2O: MP 247~248°C (dec) (ref[1] 230~235°C (dec)); IR ν_{max}^{KBr} cm^{-1} 3440, 3320, 1690 ($CONH_2$), 1480, 1310 (N(O)=N).

Anal Calcd for $C_8H_7N_3O_4$:
C 45.94, H 3.37, N 20.09.
Found: C 46.10, H 3.36, N 20.07.

2-(Carbamoyl-*N*,*N*,*O*-azoxy)benzoic Acid (**5b**)

Into a 0°C cooled solution of **1b** (0.400 g, 2.08 mmol) in 2 ml of THF containing 0.4 ml of H_2O, hydrogen chloride gas was bubbled over 5 minutes. The reaction mixture, treated as reported for the preparation of **5a**, gave **5b** (yield 95%). The product was quantitatively recrystallized from EtOH - H_2O: MP 189~190°C (dec); IR ν_{max}^{KBr} cm^{-1} 3370, 3180 (NH_2), 1690 CO(NH_2) overlapping with 1710 CO(OH) absorption), 1490 (d), 1330 (N(O)=N).

Anal Calcd for $C_8H_7N_3O_4$:
C 45.94, H 3.37, N 20.09.
Found: C 46.10, H 3.36, N 20.07.

Acknowledgments

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