ACIVICIN (AT 125, U 42126): ISOLATION AND STRUCTURE OF AN ALKALI-INDUCED CONTAMINANT

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Screening soil cultures for antimetabolite antitumor activity[1] led to the isolation[2] of acivicin (**1a**) (Fig. 1). The unique enzyme-inhibiting and antitumor effects[3] of acivicin led to its ongoing clinical trials by the National Cancer Institute. At one time, clinical formulations of NSC 163501 consisted of freeze-dried solutions of acivicin and mannitol adjusted to pH 7 with dilute alkali prior to lyophilization. In the course of such a formulation, an NCI contractor inadvertently added 1 N rather than 0.1 N alkali to a 200 g lot of acivicin. After 1 hour at pH 12 the solution was neutralized with HCl and lyophilized and the resulting solids returned to Upjohn for recovery of the acivicin. Pure acivicin was recovered in 89% yield by previously described *tert*-butyloxycarbonyl derivatization procedures.[4] This report describes the isolation and structure of a ninhydrin-positive contaminant formed in trace amounts by the alkaline treatment.

The contaminant was detected in mother liquor residues by TLC on Whatman LK6DF silica gel plates developed with propanol - ethyl acetate - water (4: 4: 3): the contaminant was observed at Rf 0.33, acivicin at Rf 0.40, and tricholomic acid (**2a**),[5] (Fig. 1), the normal alkaline hydrolysis product of acivicin,[6] at Rf 0.16. Mother liquor residues were derivatized[4] and extracted with two portions of ethyl acetate after acidification. The first extract yielded homogeneous acivicin after acid regeneration and recrystallization. The second extract afforded 2.0 g of impure acivicin (88% purity) and a mother liquor residue (2.06 g) consisting mainly of salt along with 159 mg of acivicin and the contaminant. This residue was chromatographed on silica gel with propanol - ethyl acetate - water (4: 4: 3) and collected fractions were monitored by weight and TLC. Pooling and evaporating appropriate fractions afforded 259 mg of the Rf 0.33 contaminant as a chromatographically homogeneous residue. Earlier fractions contained acivicin and later fractions contained salts and considerably smaller amounts of other unidentified ninhydrin-positive contaminants; one of these trace contaminants moved with tricholomic acid on TLC.

Fast atom bombardment (FAB) MS indicated that the major contaminant had a MW of 320 and suggested the presence of a single Cl. FAB-MS peak match $(M+K)^+$ measured at 359.0174. $C_{10}H_{13}ClN_4O_6K$ requires 359.0161. These results and those from ^1H and ^{13}C NMR were consistent with a coupled product of acivicin and a tricholomic acid-like molecule. Observed NMR signals were consistent with all the protons and carbons of acivicin and tricholomic acid except the carbon signal for the lactam carbonyl of tricholomic acid. A new carbon signal at 160.6 ppm was so broad that it was barely discernible. Structure **3a** (Fig. 1), resulting from the amino group of one acivicin molecule displacing the chloro group of another, is consistent with the observed spectra and would afford tricholomic acid on further hydrolysis.[6] Additional support for structure **3** was obtained by SCHOTTEN-BAUMANN benzoylation of the contaminant. The chromatographic polarity, acidic-water solubility, and observed FAB-MS MW 424 of the benzoyl derivative were consistent with the monobenzoyl structure **3b** (Fig. 1), which is still an amino acid. A sample of **3b** purified to chromatographic homogeneity on silica gel resisted crystallization efforts but counter-current chromatography in the Ito Coil[7] afforded pure crystalline **3b**.

A sample (50 mg, 0.156 mmol) of the isolated contaminant reacted with benzoyl chloride in aqueous sodium hydroxide. The resulting suspension was acidified and extracted with toluene to remove benzoic acid and then with three portions of butanol. TLC densitometry on silica gel (Whatman LK6DF plate) developed with methylethylketone - acetone - 1 N acetic acid (65: 20: 15) and monitored with 230 nm UV light established the presence of a major component at Rf 0.14 in the butanol extracts. Small aliquots of the non-volatile oily residue (83 mg) from the butanol extracts were partitioned between 0.5 ml portions of the upper and lower phases of several pre-equilibrated biphasic solvent systems and the

Fig. 1. Acivicin and its hydrolysis products.

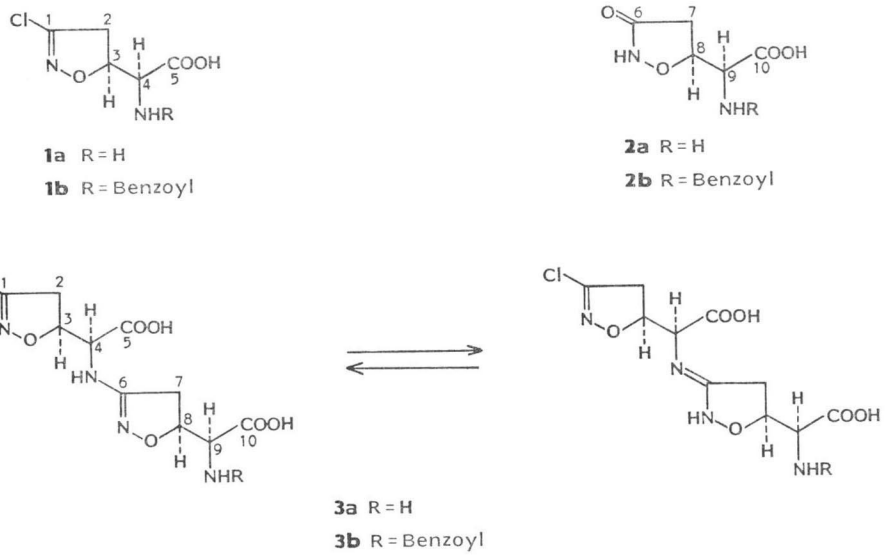

Table 1. ^{13}C NMR shifts.

Tentative assignment	Multiplicity[a]	Chemical shifts[b]				
		1a[c]	3a[c]	1b[d]	2b[d]	3b[d]
7	t		35.6		37.0	38.0
2	t	41.3	39.7	41.4		41.4
9	d		55.6		56.1[g]	55.7
4	d	57.5	60.3	55.7		59.1
8	d		77.0		81.7	79.7
3	d	81.9	82.8	82.8		82.5
m[e](2)	d			128.3	128.2	128.2
o[e](2)	d			129.2	129.2	129.1
p[e]	d			132.5	132.4	132.4
1′[e]	s			134.6	134.6	134.5
1	s	153.8	152.0	150.2		150.3
Carbonyl[e]	s			167.9	167.9	168.0
5	s	171.4	170.4	170.7		170.8
10	s		174.3		171.1	171.5
6	s		160.6[f]		172.5	159.0

[a] s: Singlet, d: doublet, t: triplet. [b] Chemical shifts in ppm relative to internal TMS on Varian XL 200. [c] In D_2O. [d] In acetone-d_6. [e] Benzoyl carbon. [f] Broad-tautomeric mixture. [g] Enol form-additional signal at 56.2 ppm.

concentration of the Rf 0.14 component in each phase was estimated by TLC densitometry. The system from chloroform - methanol - 0.2 N acetic acid (1 : 1 : 1) evenly partitioned this component between its phases and was used for counter-current chromatography in the Ito multilayer coil separator-extractor (P. C., Inc). After filling the column (2.6 mm i.d.; volume approximately 330 ml) with the upper phase as the stationary phase, the oily residue was dissolved in 8 ml of a mixture of the phases and introduced into the coil through a loop valve. Lower phase was then used as the mobile phase at a flow rate of 4.6 ml/minute while the coil was rotated at 800 rpm. After the loss of 79 ml of stationary phase (retention 251 ml or 76%), collected fractions of the mobile phase were monitored by TLC densitometry. After 444 ml of mobile phase, homo-

geneous Rf 0.14 component (**3b**) appeared in the next 185 ml of eluate. Evaporation of center-cut fractions (111 ml containing 89% of the **3b** present) left 40 mg (0.094 mmol, 60%). Crystallization from acetone - cyclohexane afforded 34 mg of **3b** as white needles. FAB-MS: $(M+H)^+$ at 425. Peak match $(M+K)^+$ measured at 463.0416. $C_{17}H_{17}ClN_4O_7K$ requires 463.0423.

Since these needles were unsuited for X-ray diffraction studies, the structure of **3b** was confirmed by additional NMR studies. Benzoyl acivicin (**1b**) prepared and purified by counter-current chromatography, using hexane - ethyl acetate - methanol - 0.2 N acetic acid (1: 2: 1: 2) with the upper phase as the mobile phase, was a clear, colorless gum. FAB-MS: $(M+H)^+$ at 283. Benzoylation of tricholomic acid afforded a complex mixture from which a crystalline monobenzoyl derivative (**2b**) was isolated by chromatography and crystallization. MS: M^+ at 264. NMR data on **1a**, **1b**, **2b**, **3a** and **3b** is summarized in Table 1. A well-defined signal at 159 ppm was assigned to carbon 6 of **3b**.

Contaminant **3a** retained only a trace of the antimicrobial activity of acivicin against *Bacillus subtilis* grown on a synthetic medium and was inactive against P388 leukemia in mice up to the highest dose tested (100 mg/kg, qd 1×5 schedule).

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