CYTOTOXIC, FUNGICIDAL NUCLEOSIDES FROM BLUE GREEN ALGAE BELONGING TO THE SCYTONEMATACEAE

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Tubercidin, toyocamycin, and the corresponding 5′-α-D-glucopyranose derivatives of the nucleosides are frequently responsible for much of the cytotoxicity and antimycotic activity associated with extracts of cultured cyanophytes belonging to the family Scytonemataceae. The 5′-α-D-glucopyranoses of tubercidin and toyocamycin, for example, are the major cytotoxic and fungicidal nucleosides in Fijian *Plectonema radiosum* and Hawaiian *Tolypothrix tenuis*, respectively.

The blue green algae provide an excellent source of new bioactive compounds[1]. Over the past 6 years we have mass cultured over 700 clonal isolates from a variety of terrestrial, freshwater and marine environments and screened hydrophilic and lipophilic extracts of these cyanophytes for cytotoxicity and antifungal activity. About 6% of the extracts show cytotoxicity at <20 μg/ml against the KB cell line (a human epidermoid carcinoma of the nasopharynx) and roughly 9% of the extracts show antifungal activity at 500 μg/disc against one or more test organisms, *viz. Aspergillus oryzae*, *Candida albicans*, *Penicillium notatum*, *Saccharomyces cerevisiae* and *Trichophyton mentagrophytes*. Several of the active hydrophilic extracts (obtained with 30% ethanol in water) show both cytotoxicity and antifungal activity and many are cyanophytes belonging to the family Scytonemataceae.

Two distinct classes of compounds are responsible for the cytotoxicity and fungicidal activity of the Scytonemataceae listed in Table 1, *viz.* scytophycin-type macrolides and tubercidin/toyocamycin-type nucleosides. Scytophycins account for the cytotoxicity and antifungal activity of *Scytonema pseudohofmanni* (strain BC-1-2)[2,3]. Tolytoxin, a scytophycin-related compound that was first isolated from field-collected *Tolypothrix conglutinata* var. *colorata*[4], is responsible for both the cytotoxicity and antifungal activity of *Scytonema mirabile* (BY-8-1) and *Scytonema ocellatum* (DD-8-1) (unpublished results). In an earlier report we showed that tubercidin (**1**) is the major cytotoxin in cultured *Tolypothrix byssoidea* (H-6-2)[5]. Tubercidin also accounts for some of the antifungal activity associated with this cyanophyte[6]. In this paper we describe the isolation and identification of cytotoxic, fungicidal nucleosides from other Scytonemataceae. Tubercidin is responsible for much of the activity in *Scytonema saleyeriense* var. *indica* (CV-14-1). Most of the activity in *Plectonema radiosum* (DF-6-1) and *Tolypothrix distorta* (BL-11-2), however, is due to tubercidin 5′-α-D-glucopyranose (**2**). Tubercidin is a minor constituent in DF-6-1 and BL-11-2, but glucoside **2** is missing in both H-6-2 and CV-14-1. Toyocamycin (**3**) is a minor constituent in BN-7-4. The major cytotoxic, fungicidal nucleoside in *Tolypothrix tenuis* (BN-7-4) is toyocamycin 5′-α-D-glucopyranose (**4**).

Tubercidin and toyocamycin were first isolated from *Streptomyces tubercidicus*[7] and *Streptomyces toyocaensis*[8], respectively. The structures were rigorously established by synthesis[9,10]. Toyocamycin

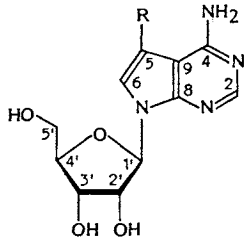
Table 1. Biological activity of extracts.

Isolate number	Extracting solvent	Identification	Origin of alga	KB MIC (μg/ml)	HL-60 (MEC μg/ml)	Dose (μg/disc)	Inhibition zone (mm)				
							AO	CA	PN	SC	TM
DD-8-1	30% EtOH	*Scytonema ocellatum*	Guam	0.5	NT	90	38	27	18	17	—
	IPA-DCM			1	NT	250	35	28	8	18	—
BY-8-1	30% EtOH	*S. mirabile*	Oahu	<18	—	500	18	16	18	—	—
BC-1-2	30% EtOH	*S. pseudohofmanni*	Oahu	0.2	—	NT					
BN-7-4	30% EtOH	*Tolypothrix tenuis*	Oahu	12	(6) +	500	10	24	34	17	24
BL-11-2	30% EtOH	*T. distorta*	Oahu	18	—	500	12	16	28	12	18
	IPA-DCM			99	NT	250	12	18	15	—	—
CV-14-1	30% EtOH	*Scytonema saleyeriense*	South Africa	2	—	500	—	11	—	14	—
	IPA-DCM			25	—	500	—	—	—	—	—
DF-6-1	30% EtOH	*Plectonema radiosum*	Fiji	14	—	500	—	12	—	19	—
	IPA-DCM			3	—	500	—	—	—	—	—
H-6-2	30% EtOH	*Tolypothrix byssoidea*	Oahu	<20	NT	500	—	—	—	13	—

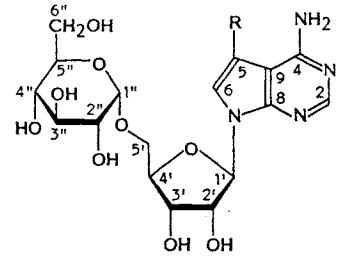
Extracting solvent was either EtOH - H_2O (3 : 7) or 2-propanol (IPA) - dichloromethane (DCM) (1 : 1). (—) Symbols indicate that no effect was observed in the bioassay. Dose for antimicrobial experiments is expressed in μg of crude extract per 7 mm filter paper disc.

MEC: Minimum effective concentration, NT: not tested.

Test organisms; AO: *Aspergillus oryzae*, CA: *Candida albicans*, PN: *Penicillium notatum*, SC: *Saccharomyces cerevisiae*, TM: *Trichophyton mentagrophytes*, KB: human epidermoid carcinoma, HL-60: human promyelocytic leukemia.



1 R = H
3 R = CN



2 R = H
4 R = CN

Table 2. Yields of nucleosides.

Isolate number	Compound (%)			
	1	**2**	**3**	**4**
DF-6-1	0.05	0.5	—	—
BL-11-2	0.02	0.5	—	—
CV-14-1	0.7	—	—	—
H-6-2	0.1	—	—	—
BN-7-4	—	—	0.005	0.7

Yields are given as weight percent compound isolated from dried alga.

vas found to possess significant antifungal activity against *Candida*, but not against other fungi. Sangivamycin[11], an analogue of toyocamycin, and tubercidin[5] have significant antitumor activity and both of these nucleosides have been evaluated clinically. Both compounds, however, have been found to be too toxic for human use.

Isolation and Structure Determination

The nucleosides were isolated from the 30% ethanol extract of each cyanophyte by reverse-phase chromatography (Table 2). The glucopyranose derivative was eluted from C-18 bonded phase silica prior to the unglucosylated nucleoside in about 0.5% yield for BL-11-2, BN-7-4, and DF-6-1. Much less tubercidin or toyocamycin (0.05~0.005%) was isolated from these three organisms. Tubercidin was isolated from CV-14-1 in about 0.7% yield.

Both glucosides showed essentially the same ^{13}C NMR spectra in the 60~100 ppm region, indicating the presence of identical sugar moieties. Moreover, each glucoside had a ^{13}C NMR spectrum that was very similar to that of the corresponding unglucosylated nucleoside. ^{1}H NMR analysis of each glucoside showed that an α-glucose was attached to C-5′, since the coupling between 1″-H and 2″-H was 3.7 Hz and couplings were observed in dimethyl sulfoxide-d_6 between exchangeable protons and ring protons in all cases except for 1″-H and the two protons on C-5′. Treatment of **2** and **4** with α-D-glucosidase confirmed the presence of an α-glucose moiety and furthermore indicated that it was D. The tubercidin and toyocamycin from enzymatic degradation of the glucosides were identical with authentic samples.

Biological Activity

Cytotoxicity (KB)

The glucosides were found to be less cytotoxic than the corresponding unglucosylated nucleosides.

Table 3. Induction of HL-60 cell adhesion.

Test agent	Concentration (nM)	Cell number		%
		Suspended	Adherent	
Negative control		5.8×10^6	1.8×10^4	0.3
TPA	5	5.3×10^5	1.0×10^5	15.9
Tubercidin (**1**)	1	7.2×10^5	3.6×10^4	4.8
	10	5.3×10^5	3.0×10^4	5.4
Toyocamycin (**3**)	1	4.9×10^5	1.2×10^4	2.4
	10	4.5×10^5	2.4×10^4	5.0
Tubercidin glucoside (**2**)	1	8.6×10^6	8.4×10^4	1.0
	10	2.1×10^6	7.7×10^4	3.5
Toyocamycin glucoside (**4**)	1	1.4×10^6	5.5×10^4	3.8
	10	7.2×10^5	6.2×10^4	7.9

Cells (1×10^6) were treated with nucleosides at the concentration indicated. Numbers of suspended and adherent cells were determined by hemacytometer counts after 96 hours incubation. Each data point represents the mean of three determinations. 12-*O*-Tetradecanoylphorbol-13-acetate (TPA) was included as a positive control.

In the KB assay, the MICs of **1** and **2** are 70[8] and 560 ng/ml, respectively, and the MICs of **3** and **4** are 60 and 300 ng/ml, respectively.

Differentiation (HL-60)

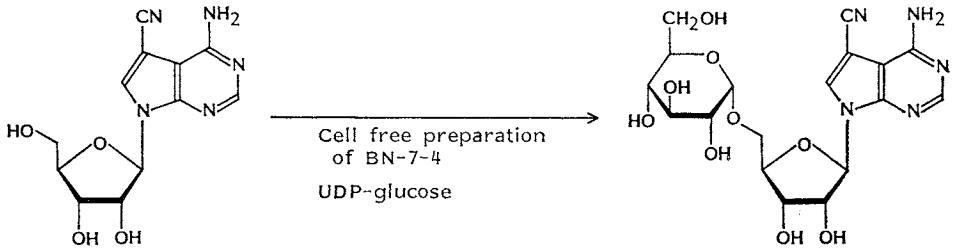
The extract from *T. tenuis* (strain BN-7-4) induced terminal differentiation in HL-60 cells (Table 3). Examination of the purified nucleosides and their respective glucosides (Table 3) indicated that all were, to some extent, capable of inducing differentiation. Similar differentiation of HL-60 has been observed in the presence of other nucleosides[12], apparently as a result of inhibition of DNA synthesis[13,14].

The range of concentration between the minimum effective dose required to initiate HL-60 differentiation and the cytotoxic dose is relatively narrow for these compounds. Concentrations of pure compound in excess of 10 nM were usually toxic, resulting in reduced differentiation. It appears likely that this toxicity was responsible for negative results when crude extracts of BL-11-2, CV-14-1, and DF-6-1 were tested in our screening assay.

Preliminary Biosynthetic Studies

Tubercidin and toyocamycin exhibit poor solubility in water which limits their usefulness as drugs. The corresponding 5′-glucopyranoses, however, are much more water soluble. Curiously, the use of this type of nucleoside derivative in drug delivery has not been described. It appears that the 5′-glucopyranose nucleoside antibiotics are quite common in the Scytonemataceae and the biogenesis of these compounds raises some interesting questions. Do the organisms share a similar or identical enzymatic process, for example one which attaches the glucose moiety to the 5′-position of the free nucleoside? To our knowledge, no such mechanism has ever been reported for nucleosides. To investigate this possibility we incubated cell free preparations of *T. tenuis* (BN-7-4) with ^{14}C-labeled glucose and ^{14}C-labeled UDP-glucose, respectively, for various lengths of time. Isolation of toyocamycin 5′-α-D-glucopyranose by HPLC and analysis with a radioactivity HPLC detector as well as liquid scintillation counting verified that glucose from [*glucose-U*-^{14}C]UDP-glucose was incorporated into toyocamycin 5′-α-D-glucopyranose (specific incorporation: 2.0% after 10 minutes at 37°C). No incorporation could be detected upon incubation with [*U*-^{14}C]glucose. These results suggest that an

Scheme 1.



enzyme or enzyme complex transfers glucose from UDP-glucose to the 5′-position of toyocamycin (Scheme 1). Experiments to isolate and characterize the UDP-glucose-toyocamycin-glucosyltransferase (named in analogy to UDP-glucose-DNA-β-glucosyltransferase EC 2.4.1.27) are currently in progress. Detailed studies on the biosynthesis of toyocamycin and tubercidin have been reported[15,16].

Experimental

^{1}H NMR spectra were obtained at 300 MHz in DMSO-d_6 and ^{13}C NMR spectra at 75 MHz in DMSO-d_6. Chemical shifts are reported in δ units (ppm) relative to the solvent as internal standard for both ^{1}H (2.52 ppm) and ^{13}C (39.5 ppm).

Culture Conditions

Clonal isolates of BL-11-2 (Palolo Valley, Oahu), BN-7-4 (Sacred Falls, Oahu), CV-14-1 (Sabie River, South Africa), and DF-6-1 (Nadi, Fiji) were prepared by repeated subculture on solidified medium and mass cultivated in liquid medium, using the procedure described for *Hapalosiphon fontinalis*[17]. Each alga was harvested by filtration after 3~5 weeks and yields of lyophilized cells typically amounted to 0.4~0.7 g/liter of culture. The cyanophytes were identified as *T. distorta* Kutzing ex Bornet & Flahault (BL-11-2), *T. tenuis* (Kutzing) Johs. Schmidt em. (BN-7-4), *S. saleyeriense* var. *indica* Bharadwaja (CV-14-1), and *P. radiosum* (Schiederm.) Gomont (DF-6-1) according to the system of DESIKACHARY[18]. All are members of the Scytonemataceae (Nostocales, Cyanophyceae, Cyanophyta), a family comprised of filamentous organisms with a firm sheath and false branches. Heterocysts are present in the genera *Scytonema* and *Tolypothrix*, but are absent in the genus *Plectonema*.

Preliminary Examination of Algae for Cytotoxins and Fungicides

One g of freeze-dried alga was extracted with 100 ml of EtOH - H_2O (3:7), (7:3) or dichloromethane - 2-propanol (1:1) for 12 hours. The insoluble portion was removed by filtration or centrifugation and the extract assayed for KB cytotoxicity and fungicidal activity.

Forty mg portions of the extract were chromatographed on a 2×0.9-cm column of C-18 BondElut (500 mg, Analytichem International). The extract was introduced onto the column with water or water containing a small amount of EtOH. Elution was carried out with 10 ml quantities of H_2O (fraction 1), MeOH - H_2O (1:9, fraction 2), (3:7, fraction 3), (1:1, fraction 4), (3:1, fraction 5), (9:1, fraction 6), MeOH (fraction 7), acetonitrile (fraction 8), EtOAc (fraction 9), and hexane (fraction 10). Each fraction was examined on a C-18 TLC plate with MeOH - H_2O (3:2) and then with MeOH if material remained at the base line (plates developed with 1% phosphomolybdic acid in EtOH). The first seven fractions from BondElut were analyzed by reverse-phase HPLC on a 100×4.6-mm C-18 column, at 25°C and using a flow rate of 1 ml/minute. With fractions 1~3, each chromatogram was developed isocratically with 15% MeOH for 20 minutes, further developed by a linear gradient to 100% MeOH over 10 minutes, and finally the remainder of the chromatogram flushed from the column with a 10-minute MeOH wash. With fractions 4~7, the procedure was essentially the same, except that 50 and 75% MeOH were used for isocratic development of the chromatograms of fractions 4

and 5, respectively, and an additional wash with acetonitrile was used to terminate the chromatographies of fractions 6 and 7. Fractions 8~10 were not analyzed by C-18 HPLC. Bioassays indicated that fractions 1, 2, 4 and 5 were both cytotoxic and antifungal. Roughly one-half of the activity of the crude extract was found in fractions 1 and 2 and the remaining one-half in fractions 4 and 5. Tubercidin and toyocamycin nucleosides were detected in fractions 1 and 2 using a UV diode array detector to analyze the various HPLC peaks. With the HPLC conditions described above, the retention times for tubercidin 5′-α-D-glucopyranose (**2**), tubercidin (**1**), toyocamycin 5′-α-D-glucopyranose (**4**), and toyocamycin (**3**) were found to be 4, 7, 8, and 9 minutes, respectively. The tubercidin nucleosides were characterized by two UV maxima at 210 and 265±5 nm (relative intensities 2:1) and the toyocamycin nucleosides by three UV maxima at 210, 230, and 270±5 nm (relative intensities 2:0.75:1). The cytotoxic and antifungal substances in fractions 4 and 5 were not fully characterized. HPLC and UV analysis indicated that tubercidin and toyocamycin-type compounds were not present in fractions 4 and 5.

Determination of Biological Activity

Antitumor activity *in vitro* using the human epidermoid carcinoma cell line (KB; ATCC CCL 17) was determined using the procedure of GERAN *et al.*[19]. Antifungal activity was determined using an agar diffusion method[20], and previously described test organisms[21].

Introduction of differentiation of the human promyelocytic leukemia cell line (HL-60; ATCC CCL 240) was detected by a screening procedure involving treatment of radiolabeled HL-60 cells with aliquots of crude extract. Induction of differentiation resulted in attachment of the HL-60 cells to the surface of the culture vessel[22,23]. Following incubation for periods up to 48 hours, unattached cells were removed by aspiration. The presence of adherent (differentiated) cells was then determined by liquid scintillation counting. Determination of the degree of differentiation induced by pure compounds was determined by the method of ROVERA *et al.*[24], in which unlabeled cells were treated with various doses of test agent for various lengths of time. After incubation, the numbers of adherent and non-adherent cells were determined by hemacytometer counts.

Isolation of Tubercidin Nucleosides

Freeze-dried BL-11-2 (11.4 g) was extracted with 570 ml of EtOH - H_2O (3:7) followed by 570 ml of dichloromethane - 2-propanol (1:1). The aqueous ethanolic extract was reduced to about 50 ml. A 1-ml aliquot of this concentrate, which contained 37 mg of extract, was applied to a C-18 BondElut cartridge that had been preconditioned with water. Elution with 9 ml H_2O, 5 ml of MeOH - H_2O (1:4), and 5 ml of MeOH yielded 28, 3, and 4 mg fractions. The 20%-MeOH fraction, which contained most of the bioactivity, was further separated by isocratic HPLC on a C-18 Econosphere (Alltech, 10 μm) column (10×250 mm) to give 1.1 mg of tubercidin-5′-α-D-glucopyranose (**2**) (retention time (t_R) 20.5 minutes; 0.5% yield based on the dried weight of the alga) and <0.1 mg of tubercidin (**1**) (t_R 35.0 minutes). An 8:92 mixture of MeOH and 0.1 N aqueous ammonium acetate buffered at pH 7.0 was used as the eluant (flow rate of 2 ml/minute and continuous UV monitoring of the effluent at 268 nm). Compound **2** had the following properties: $[\alpha]_D^{25}$ +10° (*c* 0.01, H_2O); UV $\lambda_{max}^{H_2O}$ nm ($E_{1cm}^{1\%}$) 268 (9,300); IR (KBr) ν_{max} cm^{-1} 1650, 1595, 1555, 1400 (s); fast atom bombardment mass spectrum (FAB-MS) *m/z* 429 (M+H); high resolution FAB-MS *m/z* 429.1603 (calcd for $C_{17}H_{25}N_4O_9$, 429.1595); ^1H NMR (DMSO-d_6) δ 8.03 (1H, s, 2-H), 7.69 (1H, d, *J*=3.7 Hz, 6-H), 6.95 (2H, br s, 4-NH_2), 6.53 (1H, d, *J*=3.7 Hz, 5-H), 6.12 (1H, d, *J*=7.0 Hz, 1′-H), 5.19 (2H, d, *J*=4.8 Hz, 2″-OH and 3′-OH), 5.10 (1H, d, *J*=4.8 Hz, 2′-OH), 4.93 (1H, d, *J*=3.8 Hz, 3″-OH), 4.85 (1H, d, *J*=4.6 Hz, 4″-OH), 4.70 (1H, d, *J*=3.5 Hz, 1″-H), 4.50 (1H, d, *J*=4.7 Hz, 6″-OH), 4.41 (1H, td, *J*=6.3, 4.8 and 4.8 Hz, 2′-H), 4.09 (1H, td, *J*=4.9, 4.9 and 2.1 Hz, 4′-H), 4.05 (1H, m, *J*=4.6, 4.4 and 2.3 Hz, 3′-H), 3.76 (1H, dd, *J*=3.1 and −11.1 Hz, 5′-H), 3.64 (1H, ddd, *J*=4.8, 1.2 and −10.9 Hz, 6″-H), 3.45 (1H, m, 4″-H), 3.44 (1H, m, 6″-H), 3.41 (1H, dd, *J*=4.9 and −11.1 Hz, 5′-H), 3.34 (1H, m, *J*=8.1 and 1.2 Hz, 5″-H), 3.26 (1H, m, *J*=9.3, 4.8 and 3.6 Hz, 2″-H), 3.10 (1H, td, *J*=9.3, 9.3 and 3.8 Hz, 3″-H); ^{13}C NMR (DMSO-d_6) δ 157.3 (C-4), 151.5 (C-2), 150.6 (C-8), 122.2 (C-6), 103.0 (C-9), 99.8 (C-5), 98.7 (C-1″), 85.8 (C-1′), 83.0 (C-4′), 74.0 (C-2′), 73.4 (C-4″), 72.9 (C-5″), 71.8 (C-2″), 71.1 (C-3′), 70.2 (C-3″), 67.2 (C-5′), 60.9 (C-6″). Freeze-dried DF-6-1 (10 g) was processed as described below for BN-7-4 to give

73.8 mg of **2** (t_R 20.5 minutes) and <0.1 mg of **1** (t_R 35.0 minutes).

Isolation of Toyocamycin Nucleosides

The freeze-dried BN-7-4 (10 g) was extracted with 600 ml of EtOH - H_2O (7 : 3). The filtered extract was evaporated and the residual solid in 10 ml of water was applied to a 2×50-cm column of Amberlite XAD-2 resin. The column was washed with 500 ml of water followed by 400 ml of EtOH - H_2O (1 : 9). The cytotoxic agent was then eluted from the column with 400 ml of EtOH - H_2O (4 : 6). The active fraction was prepared for HPLC by passing the material through a 7 (diameter)×1-cm (depth) column of C-18 bonded silica (Baker, 40 μm) with 500 ml of MeOH - H_2O (3 : 17). Final purification was achieved by isocratic, reverse-phase HPLC on a Whatman Partisil M9 ODS-2 column using MeOH - H_2O (3 : 17) as the eluant (flow rate 2 ml/minute). The major peak (t_R 48 minutes) was collected and lyophilized to give 68 mg of toyocamycin 5′-α-D-glucopyranose (**4**) (0.7% yield) as a white amorphous powder: $[\alpha]_D^{25}$ +2.1° (*c* 0.03, H_2O); UV $\lambda_{max}^{H_2O}$ nm ($E_{1cm}^{1\%}$) 278 (10,000), 230 (7,400); IR (KBr) ν_{max} 2230 cm^{-1} (CN); electron impact mass spectrum (EI-MS) *m/z* 159 (cleavage at N-5 – C-1′); field desorption mass spectrum (FD-MS) *m/z* 453; FAB-MS *m/z* 454 (M+H), 476 (M+Na), 929 (2M+Na); high resolution FAB-MS *m/z* 454.1567 (calcd for $C_{18}H_{24}N_5O_9$, 454.1574); FAB-MS-MS *m/z* 454→437 (M−OH), 292 ($C_{12}H_{14}N_5O_4$), 160 ($C_7H_6N_5$); ^{1}H NMR (DMSO-d_6) δ 8.70 (1H, s, 6-H), 8.21 (1H, s, 2-H), 6.83 (2H, br s, 4-NH_2), 6.18 (1H, d, *J*=7.0 Hz, 1′-H), 5.46 (1H, d, *J*=6.7 Hz, 2′-OH), 5.35 (1H, d, *J*=5.1 Hz, 2″-OH), 5.33 (1H, d, *J*=4.6 Hz, 3′-OH), 4.98 (1H, d, *J*=5.2 Hz, 3″-OH), 4.80 (1H, d, *J*=4.4 Hz, 4″-OH), 4.72 (1H, d, *J*=3.5 Hz, 1″-H), 4.53 (1H, t, *J*=5.8 Hz, 6″-OH), 4.41 (1H, td, *J*=7.0, 6.7 and 4.6 Hz, 2′-H), 4.13 (1H, m, 4′-H), 4.10 (1H, td, *J*=4.6, 4.6 and 1.6 Hz, 3′-H), 3.80 (1H, dd, *J*=2.4 and −11.0 Hz, 5′-H), 3.64 (1H, ddd, *J*=5.8, 1.2 and −11.3 Hz, 6″-H), 3.5 (1H, 6″-H), 3.46 (1H, td, *J*=9.2, 9.2 and 4.5 Hz, 4″-H), 3.43 (1H, dd, *J*=2.2 and −11.0 Hz, 5′-H), 3.32 (1H, m, 2″-H), 3.3 (1H, m, 5″-H), 3.09 (1H, td, *J*=9.2, 9.2 and 5.3 Hz, 3″-H) (all coupling constants between non-exchangeable protons determined after addition of a trace of acid); ^{13}C NMR (DMSO-d_6) δ 156.9 (C-4), 153.5 (C-2), 150.7 (C-8), 132.5 (C-6), 115.3 (CN), 101.0 (C-9), 98.5 (C-1″), 86.4 (C-1′), 84.0 (C-4′), 83.6 (C-5), 74.9 (C-2′), 73.5 (C-4″), 73.0 (C-5″), 71.7 (C-2″), 71.2 (C-3′), 70.2 (C-3″), 66.9 (C-5′), 60.9 (C-6″).

A minor peak which passed from the ODS column above at t_R 62 minutes was collected and evaporated to give 0.5 mg of toyocamycin (**3**) (0.005%). ^{1}H NMR, ^{13}C NMR, and HPLC (coinjection with an authentic sample) analyses established the identity of this nucleoside; ^{13}C NMR (DMSO-d_6) δ 157.1 (C-4), 153.8 (C-2), 150.4 (C-8), 132.7 (C-6), 115.6 (CN), 101.6 (C-9), 88.2 (C-1′), 85.7 (C-4′), 83.4 (C-5), 74.5 (C-2′), 70.4 (C-3′), 61.4 (C-5′).

Enzymatic Hydrolysis of Tubercidin and Toyocamycin 5′-α-D-Glucopyranoses with α-D-Glucosidase

A solution of 1.0 mg of **2** and 0.46 mg of α-D-glucosidase (Sigma) in 0.5 ml 0.1 N ammonium acetate buffer (pH 6.8) was incubated in a water bath at 36°C for 5 hours. Aliquots (15 μl) were taken from the reaction mixture at various time intervals over the 5 hours period and analyzed by HPLC on a 150×4.6-mm column of C-18 Econosphere (5 μm), using MeOH - 0.1 N ammonium acetate buffer (pH 7.0) (8 : 92) as the eluant, a flow rate of 1.5 ml/minute, and a UV monitor set at 270 nm. Tubercidin and **2** had t_R values of 10.7 and 6.9 minutes, respectively. The results of the hydrolysis are shown in Fig. 1. The formation of free α-D-glucose was detected semiquantitatively by Tes-Tape (Eli Lilly)[25].

A solution of 5 mg of **4** and 0.3 mg of α-D-glucosidase in 10 ml of water buffered at pH 6.8 was incubated for 50 minutes at 37°C. ^{1}H NMR analysis indicated that 70% of the glucoside had been degraded to toyocamycin. The presence of α-D-glucose in the reaction mixture was detected with Tes-Tape (color change from yellow to green).

Biosynthesis of Toyocamycin 5′-α-D-Glucopyranose

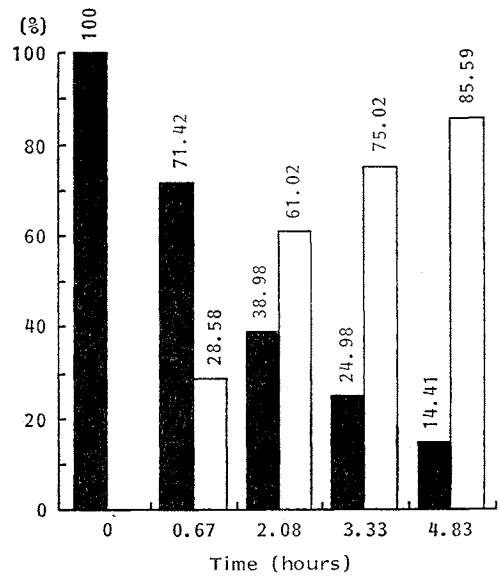
A 4-liter culture of BN-7-4 was harvested after 20 days incubation by filtration through Whatman No. 4 paper. The entire preparation of the cell-free extract was carried out at temperatures below 5°C. The cells were first washed with 0.1 M phosphate buffer (pH 7.4) then suspended in a mixture

consisting of 100 ml H_2O, 900 mg Tricine (*N*-tris(hydroxymethyl)methylglycine), 1.0 g Bovine Serum Albumin (Cohn Fraction 5), and 50 mg Soybean Trypsin Inhibitor (pH 7.4). Glass beads (100 g; 175 mesh) were added to form a thick slurry, which was homogenized (Waring-type blender) for 6 minutes in pulses of 30 seconds and intermittent cooling periods.

The slurry was centrifuged at $1,000 \times g$ to remove glass beads and the supernate was then centrifuged at $40,000 \times g$ for 50 minutes. The second supernate (20 ml) was incubated with 4.36 mg of 1 μCi UDP-glucose and 4 mg of toyocamycin for 1 hour at 37°C in a constant temperature water-bath/shaker. The mixture was then passed through a C-18 BondElut cartridge. The cartridge was then washed with water until no radioactivity was detectable in the washings. The glucopyranose was removed from the cartridge with 10 ml of MeOH - H_2O (15 : 85). Final purification was achieved by reverse-phase HPLC as described above and the pure glucopyranose (1.1 mg) was analyzed for radioactivity by liquid scintillation counting and analytical HPLC (C-18, 5 μm, 4.6×150 mm, MeOH - H_2O (15 : 85) at 0.5 ml/minute, t_R (UV) 3.6 minutes) using UV and radioactivity (liquid scintillator flowcell) detection.

Fig. 1. Enzymatic hydrolysis of tubercidin 5′-α-D-glucopyranose with α-D-glucosidase, monitored by HPLC at 270 nm.

■ Glucose-tubercidin, □ tubercidin.



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