SYNTHESIS AND CHARACTERIZATION OF A NOVEL INHIBITOR OF AN AMINOGLYCOSIDE-INACTIVATING ENZYME

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A novel low-molecular weight inhibitor of an aminoglycoside-inactivating enzyme, initially isolated from fermentation broths of *Streptomyces neyagawaensis*, was determined to be 7-hydroxytropolone. Its structure was confirmed by synthesis. *In vitro* synergy was demonstrated between 7-hydroxytropolone and certain aminoglycosides against bacteria which were resistant to those aminoglycosides by virtue of a 2″-*O*-adenylyltransferase. The synthesis and characterization of some analogs of 7-hydroxytropolone is also described.

Inhibition of an aminoglycoside inactivating enzyme, 2″-*O*-adenylyltransferase, by 7-hydroxytropolone has been reported recently.[1~3] In this paper, the structure, synthesis and antimicrobial evaluation of 7-hydroxytropolone and several closely-related analogs are described.

Identification of 7-Hydroxytropolone

7-Hydroxytropolone was found initially in the fermentation broths of *Streptomyces neyagawaensis* as an antibiotic with a broad spectrum of antimicrobial activity.* It was originally purified from crude concentrate by countercurrent distribution (CCD), using ethyl acetate - hexane - ethanol - water (8: 4: 6: 7) as the solvent system; crystallization of the product from the organic phase conveniently occurred during the purification procedure. Subsequent work showed that the desired product could be extracted directly from the fermentation broth with diethyl ether and then crystallized from the concentrated organic extract.

The crystalline product gave a parent ion at *m/z* 138 (EIMS) which high resolution measurements best matched with the empirical formula $C_7H_6O_3$. Proton and C-13 NMR spectra indicated a symmetrical molecule of aromatic character (see Table 1). UV and titration studies indicated the presence of acidic functional groups, most likely phenolic in nature, while the infrared spectrum showed the presence of a highly conjugated carbonyl group. This information suggested a symmetrical hydroxytropolone, probably 7-hydroxytropolone, as the structure of our fermentation product. The near identity of the melting point and proton NMR spectrum to those reported for 7-hydroxytropolone added further support for the proposed structure.[5,6]

Synthesis of 7-Hydroxytropolone

In order to confirm the proposed structure of the fermentation product and to provide more material for microbiological evaluation, the synthesis of 7-hydroxytropolone was carried out following a procedure starting from readily-available tropolone, as outlined by NOZOE, *et al.*[6]; however, the purification steps were modified in order to utilize countercurrent distribution methods which had been so useful in

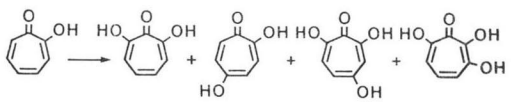
* After completion of our work, 7-hydroxytropolone was reported as a fermentation product of a *Pseudomonas* species.[4]

Table 1. Physico-chemical properties of tropolone and its 7-hydroxyl, 5-hydroxyl, 3,7-dihydroxyl and 4,7-dihydroxyl derivatives.

Compound	7-HT	5-HT	3,7-Di-HT	4,7-Di-HT*	Tropolone
Formula	$C_7H_6O_3$	$C_7H_6O_3$	$C_7H_6O_4$	$C_7H_6O_4$	$C_7H_6O_2$
Mp (°C)	136~138	244~247	238~240	215~216	51~54
Parent ion m/z (EIMS)	138	138	154	154	122
^{13}C NMR (δ, DMSO-d_6)	120.3, 128.5 160.1, 168.7	124.0, 125.8 158.0, 167.5	117.6, 127.2 156.5, 156.8	111.7, 115.0 123.8, 151.9 160.2, 162.1 163.4	124.3, 127.9 137.2, 171.7
^1H NMR (δ, DMSO-d_6)	Multiplet 7.0~7.4	AB quartet 6.89, 7.00 7.11, 7.22	Multiplet 7.01	6.67(H5, d of d) 6.99(H3, d, J=2.5) 7.26(H6, d, J=11)	Multiplet 6.9~7.5
UV λ_{max}^{EtOH} nm (log ε)	244 (4.60) 324 (3.83) 365 (3.87) 375 (3.94)	234 (4.30) 333 (3.92) 374 (3.86) 390 (3.83)	262 (4.65) 330 (3.66) 348 (3.68) 360 (3.66)	256 (4.45) 329 (3.74) 368 (3.70) 390 (3.68)	235 (4.35) 319 (3.86) 351 (3.72) 367 (3.68)
UV (basified)	258 (4.47) 336 (4.10) 412 (4.00)	234 (4.35) 362 (4.15) 398 (4.03)	285 (4.44) 350 (3.81)	284 (4.37) 340 (3.80)	239 (4.48) 332 (4.25) 398 (4.18)

* This product contained about 15 percent of 3,7-Di-HT, which was not removed by crystallization.

Fig. 1. Synthesis of 7-hydroxytropolone.



7-HT 5-HT 4,7-Di-HT 3,7-Di-HT

isolation of the natural product. As a result, four products were readily separated from the complex reaction mixture, one of which was a previously unreported dihydroxytropolone.

Persulfate oxidation of tropolone followed by separation of the products using CCD (chloroform - toluene - methanol - water, 15:15:23:7) and crystallization yielded 7-hydroxytropolone in moderate yield along with the 5-hydroxyl isomer and the 3,7-dihydroxyl and 4,7-dihydroxyl derivatives of tropolone. The physico-chemical properties of these products are compared with those of tropolone in Table 1. The synthetic 7-hydroxytropolone was identical in all respects to the fermentation-derived product. 5-Hydroxytropolone and 4,7-dihydroxytropolone were identified by comparison of their physico-chemical properties (Table 1) with literature data.[5,7,8] The structures of 7-hydroxytropolone

Fig. 2. ORTEP drawing of 7-hydroxytropolone with bond distances (σ=±0.003 Å) for nonhydrogen atoms.

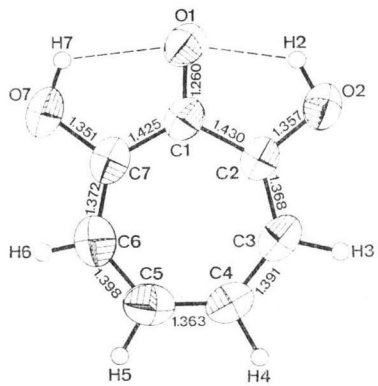
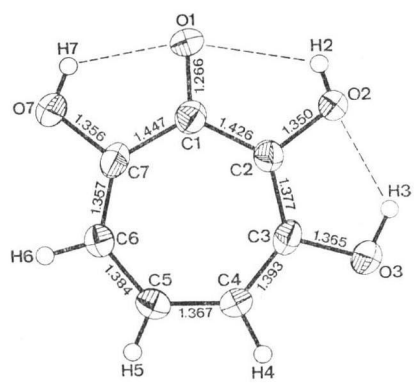


Fig. 3. ORTEP drawing of 3,7-dihydroxytropolone with bond distances (σ=±0.005 Å) for nonhydrogen atoms.



and the previously unreported 3,7-dihydroxytropolone* were rigorously established by X-ray crystal structure analysis; this information on molecular structure could be useful for model enzyme studies. Single crystals of 7-hydroxytropolone were obtained as yellow needles from diethyl ether while 3,7-dihydroxytropolone yielded dark red prisms when crystallized from ethyl acetate. Fig. 2 is an ORTEP drawing of the crystal structure of 7-hydroxytropolone while the crystal structure of 3,7-dihydroxytropolone is depicted in Fig. 3.

Antimicrobial Activity

The hydroxylated derivatives of tropolone possessed broad antimicrobial activity, as does tropolone

Table 2. Comparative antimicrobial activity of hydroxyl derivatives of tropolone[a].

	7-HT	5-HT	3,7-Di-HT	4,7-Di-HT	Tropolone
Staphylococcus aureus X1	27	NA[b]	30	25	24
Bacillus subtilis X12	19	tr[c]	30	23	24
Micrococcus luteus X186	18	15	30	13	10
Escherichia coli X161	18	16	24	20	19
Proteus vulgaris X45	17	tr	30	20	15
Serratia marcescens X99	NA	tr	16	11	15
Pseudomonas aeruginosa X48	tr	NA	16	10	14
Candida albicans A26	19	NA	42	18	22
Neurospora crassa 846	23	NA	12	10	23
Trichophyton mentagrophytes A23	17	NA	22	15	25

[a] Zone diameter (mm) in an agar diffusion assay around a 6-mm disc containing approximately 25 μg of compound.
[b] No zone of inhibition.
[c] Trace zone of inhibition (<8 mm).

Table 3. *In vitro* synergy studies of 7-hydroxytropolone (7-HT) and aminoglycosides (AG)[a].

	MIC values (μg/ml)[b]					
	E. coli X560		*K. pneumoniae* OK8		*K. pneumoniae* MC2	
	AG	AG/7-HT	AG	AG/7-HT	AG	AG/7-HT
Tobramycin	64	4/1	64	1/8	64	4/4
Gentamicin	2,048	64/2	64	2/8	32	1/4
Kanamycin A	>4,096	>4,096/8	512	16/8	512	256/16
Kanamycin B	>4,096	>4,096/8	128	4/8	>1,024	512/16
Dibekacin	4,096	256/2	>128	16/8	>128	16/4
Sisomicin	2,048	64/2	>32	2/8	>32	1/4
Netilmicin	32	2/2	2	0.25/8	1	0.25/8
Amikacin	16	2/4	0.5	0.5/16	2	1/4
Apramycin	64	64/8	2	1/16	4	2/8

[a] The effect of the various antibiotic combinations was determined *via* a broth-microdilution checkerboard technique, using supplemented Müeller-Hinton broth in an Ames Autotiter. The methods used were those proposed in Cumitech 6 (ASM, September, 1977).
[b] MIC of 7-HT alone was 8 μg/ml *vs. E. coli* X560, 64 μg/ml *vs. K. pneumoniae* OK8 and 32 μg/ml *vs. K. pneumoniae* MC2.

* Acetyl derivatives have been reported.[9]

itself.[10] The antibiotic activity of the hydroxylated tropolones is compared in Table 2; the activity of all of the compounds was qualitatively similar except for that of 5-hydroxytropolone, which was unexpectedly a much weaker antibiotic than were the other compounds.

Synergy Studies

When tested against bacterial strains which were resistant to aminoglycosides by virtue of a 2″-*O*-adenylyltransferase, 7-hydroxytropolone was synergistic with those aminoglycosides which were substrates for the 2″-*O*-adenylyltransferase. The results of these *in vitro* synergy studies are reported in Table 3.

Escherichia coli X560 contains both a 2″-*O*-adenylyltransferase and a 3′-*O*-phosphotransferase; as a result, kanamycin A and kanamycin B did not inhibit *E. coli* X560 in the presence of 7-hydroxytropolone because the latter did not inhibit the 3′-*O*-phosphotransferase.[3] *Klebsiella pneumoniae* MC2 likewise contains a 3′-*O*-phosphotransferase (J. HOBBS, Jr., unpublished results) and consequently was resistant to kanamycins A and B in the presence of 7-hydroxytropolone. In contrast, *K. pneumoniae* OK8, which contains the 2″-*O*-adenylyltransferase but lacks the 3′-*O*-phosphotransferase (J. HOBBS, Jr., unpublished results), was inhibited by the combination of kanamycin A or B and 7-hydroxytropolone. Clearly, inhibition of each of those enzymes which inactivate an aminoglycoside in a resistant bacterial strain is required before that resistant strain will be rendered susceptible to the particular aminoglycoside antibiotic. Unfortunately, 7-hydroxytropolone does not inhibit *O*-phosphotransferases or *N*-acetyltransferases although preliminary evidence suggests that it may inhibit *O*-adenylylation of hydroxyl groups other than those at the 2″-position.[3]

Aminoglycosides which are not substrates for the 2″-*O*-adenylyltransferase are not expected to be affected by the presence of 7-hydroxytropolone. Thus, apramycin and amikacin in combination with 7-hydroxytropolone showed only minor changes in their MIC values; these were probably due only to additive effects since a four-fold or greater reduction in the MIC value for both compounds was not achieved.

A modest enhancement of the efficacy of tobramycin was also demonstrated against experimental infections in mice induced with bacteria resistant to tobramycin by virtue of a 2″-*O*-adenylyltransferase. Against an infection caused by *K. pneumoniae* OK8, a three-fold decrease of the ED_{50} value for tobramycin was observed using a 1 : 1 combination of tobramycin and 7-hydroxytropolone (Table 4). Against an infection caused by *K. pneumoniae* MC2, the effect was less dramatic; however, a 1 : 1 combination of tobramycin and 7-hydroxytropolone may not have been the most effective ratio of the two compounds in this case. Nevertheless, the *in vivo* results are clearly parallel to the *in vitro* results, demonstrating potentiation of tobramycin by 7-hydroxytropolone against these resistant strains.

Table 4. *In vivo* synergy studies of 7-hydroxytropolone and aminoglycosides.

Treatment	ED_{50} values (mg/kg×2) in mice[a]	
	Infecting organism	
	K. pneumoniae OK8	*K. pneumoniae* MC2
Tobramycin	63	12
7-Hydroxytropolone	>100	89
1 : 1 Combination of tobramycin and 7-HT	23	9

[a] Compound(s) administered subcutaneously 1 and 5 hours post-infection.

Analogs of 7-Hydroxytropolone

Although a large number of compounds were tested for inhibition of 2″-*O*-adenylyltransferase after

the structure of 7-hydroxytropolone was elucidated, very few of these showed any significant enzyme-inhibitory activity. Non-tropolone compounds were completely ineffective as inhibitors; among those tested were pyrogallol; 2,6-dihydroxybenzoic acid, -benzaldehyde and -acetophenone; 2,3-dihydroxybenzoic acid, -benzaldehyde and -pyridine; 2,2′-dihydroxybenzophenone, -biphenyl and -anthraquinone; tetrahydroxybenzoquinone, terreic acid, squaric acid; 1,3-dihydroxyacetone, tartaric acid, ascorbic acid and EDTA. Tropolone derivatives which lacked a 7-hydroxyl group were also ineffective as inhibitors; these included purpurogallin, sepedonin, stipitatonic acid, colchicine and 7-bromo, -nitro, -amino, -methylamino, -cyano and -aminomethyl-tropolone.

The synthesis of ring-substituted-7-hydroxytropolone compounds was then investigated. 3-Allyltropolone was prepared by literature procedures,[11] but persulfate oxidation yielded only 5-hydroxy-3-allyltropolone and none of the desired 7-hydroxyl isomer. In order to block oxidation at C-5, 5-(3,3-dimethylallyl)tropolone was prepared;[12] in this case, persulfate oxidation successfully yielded the 7-hydroxyl derivative, identified by comparison of spectral data with that of 4-isopropyl-7-hydroxytropolone.[13] Inhibition of the 2″-*O*-adenylyltransferase by both 4-isopropyl and 4-(3,3-dimethylallyl)-7-hydroxytropolone indicated that the tropolone ring could be substituted by short carbon chains without loss of inhibitory activity.[3]

Experimental

Preparation of 7-Hydroxytropolone

Tropolone (25 g, 0.2 mmole) was dissolved in a solution of potassium hydroxide (33.6 g) in water (360 ml) and this solution was treated dropwise with a freshly-prepared solution of potassium persulfate (60 g, 0.22 mmole) in water (1,200 ml) over a 1.5-hour period while maintaining the reaction temperature at 15~20°C. The red solution was stirred overnight at ambient temperature. The dark red reaction mixture was brought to pH 4 with 1 N sulfuric acid and extracted with ether (2×800 ml) to remove unreacted tropolone. The aqueous layer was further acidified to pH 1.9 with 1 N sulfuric acid and then warmed on a steam bath for 1 hour with occasional swirling of the reaction solution. After cooling to room temperature, the solution was brought to pH 4.2 with 5 N sodium hydroxide and was extracted with ether (3×1,000 ml). The combined ether extracts were dried (sodium sulfate), filtered and evaporated under reduced pressure to give a yellow-brown solid (6.4 g) (*Caution*: The crude concentrate or purified products may cause severe chemical burns or rash.[14]). The crude product was separated in a Craig countercurrent distribution apparatus, employing 200 transfers with a solvent system of chloroform - toluene - methanol - water (15: 15: 23: 7). The CCD tubes were analyzed by careful evaporation of aliquots from each tube and weighing of the residues; in this manner, a weight distribution profile was established for the 200 tubes.

From tubes 65~95 (K=0.66) was obtained 3.8 g of 7-hydroxytropolone, which was further purified by crystallization from ether; three crops of crystalline 7-hydroxytropolone were isolated (1.7, 0.8, 0.3 g). From tubes 120~140 (K=1.85) was obtained 0.2 g of red-brown solid which was crystallized from ethyl acetate; 58 mg of dark red crystals were obtained (second crop, 56 mg) which were identified as 3,7-dihydroxytropolone by X-ray crystal structure analysis (*vide infra*). From tubes 145~165 (K=3.4) was obtained 0.7 g of brown solid; trituration of this material with ether left 0.5 g of insoluble brown powder which was crystallized from ethyl acetate, yielding 328 mg of 5-hydroxytropolone. A small amount (69 mg) of 5-hydroxytropolone was also obtained from crystallization of the ether triturate; further concentration of the ether triturate then yielded 84 mg of crude 4,7-dihydroxytropolone which was further purified by crystallization from ethyl acetate.

Preparation of 4-Dimethylallyl-7-hydroxytropolone

5-Dimethylallyltropolone[12] (390 mg, 2.0 mmole) was dissolved in a solution of potassium hydroxide (660 mg, 10.0 mmole) in water (10 ml) and treated dropwise over a 45-minute period with a freshly-

prepared solution of potassium persulfate (594 mg, 2.2 mmole) in water (10 ml) while maintaining the reaction temperature at 15~20°C. After stirring overnight at room temperature, the solution was adjusted to pH 4.2 with 1 N sulfuric acid and extracted with ether (4×50 ml). The aqueous solution was adjusted to pH 1.9 with 1 N sulfuric acid and heated for 1 hour on a steam bath. After cooling to room temperature, the solution was adjusted to pH 4.2 with 1 N sodium hydroxide and was extracted with ether. The extract was dried (sodium sulfate), filtered and evaporated under reduced pressure to yield 100 mg of brown oil which solidified upon standing. Sublimation of this material overnight (60°C, 0.1 mmHg) yielded 33 mg of 4-dimethylallyl-7-hydroxytropolone as yellow waxy plates.

The Crystal Structures of 7-Hydroxytropolone and 3,7-Dihydroxytropolone

7-Hydroxytropolone crystallizes in the space group $P2_1/n$ with four molecules in a unit cell having the dimensions: $a=5.057\pm0.001$Å, $b=9.495\pm0.001$Å, $c=13.332\pm0.002$Å and $\beta=97.03\pm0.01°$. The X-ray diffraction intensities of 971 reflections, of which 847 were considered observed (greater than 3 sigma above background) were measured on a four-angle automated diffractometer using copper radiation. The structure was solved by direct methods and refined by the least-squares method to an R value of 0.048. The final refinement included anisotropic temperature parameters for all atoms except hydrogen, which were all found from a difference Fourier map and included with isotropic temperature factors.

3,7-Dihydroxytropolone crystallizes in the space group $P2_1/n$ with four molecules in a unit cell having the dimensions: $a=21.388\pm0.006$Å, $b=5.033\pm0.001$Å, $c=5.835\pm0.002$Å and $\beta=95.91\pm0.02°$. The intensity data were collected and the structure solved and refined as above. The final R value was 0.043 for the 831 observed reflections out of a total of 975 measured.

ORTEP plots and bond distances for the molecules are shown in Figs. 2 and 3. Tables of atomic parameters, as well as bond distances and angles, have been deposited with the Crystallographic Data Centre.

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