PREPARATIVE RESOLUTION OF AN ACTINOMYCIN COMPLEX BY COUNTERCURRENT CHROMATOGRAPHY IN THE ITO COIL PLANET CENTRIFUGE

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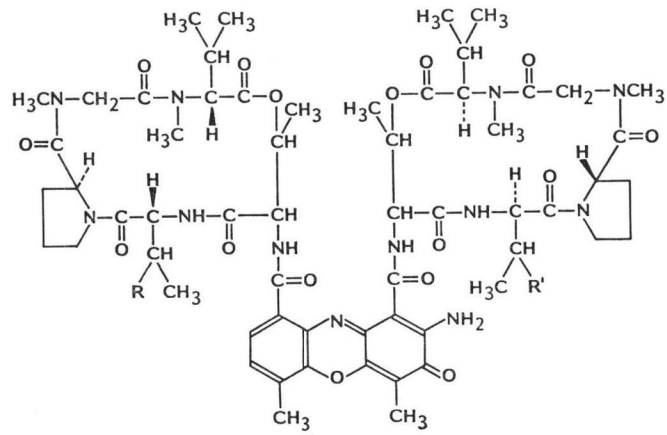
Fermentation screening cultures frequently produce mixtures of *in vitro* and *in vivo* active components. Antitumor antibiotics such as the actinomycins, displaying potent *in vitro* and *in vivo* activity, could easily mask the presence of a less visible but potentially interesting co-produced novel antibiotic. The detection and eventual isolation of such an antibiotic requires the initial efficient isolation of the potent activities present. The high resolving power and total recovery provided by countercurrent chromatography (CCC) in the Ito coil[1] prompted our efforts to isolate the actinomycins by such methods as a prelude to searching for potentially novel activities. The availability of comprehensive reviews on the actinomycins[2] and the techniques used for separating actinomycin mixtures[3] limits the necessary background for this note. Structural considerations illustrate the non-trivial nature of resolving a naturally-occurring actinomycin complex into its components (Fig. 1). Actinomycin C_2 contains a single methylene group more than actinomycin C_1 (actinomycin D) and a single methylene group less than actinomycin C_3.

The actinomycin C complex has been conveniently resolved by reversed-phase high-pressure liquid chromatography[4]; however, potentially novel co-produced activities could easily be lost through binding or deactivation by a solid chromatographic adsorbent. Historically, the most effective methods for resolving actinomycin mixtures have utilized partition systems having an aq phase containing a solubilizing agent[2,3]. Countercurrent distribution provided sufficient resolution but such methods are sufficiently time-consuming and inconvenient to limit their application.

The actinomycin C complex was isolated in the course of isolating the *in vivo* active components of cultures from multi-endpoint screening[5]. BuOH extracts from both clarified broth and mycelium were upgraded by reverse phase chromatography on C18 (Waters prep C18) with aq MeOH affording essentially pure complex. Initially, aliquots of the complex were partitioned between 1 ml portions of the phases of several equilibrated biphasic solvent systems.

Fig. 1. Structures of actinomycins C_1, C_2 and C_3.



Actinomycin	R	R′	Molecular formula	MW
C_1	CH_3	CH_3	$C_{62}H_{86}N_{12}O_{16}$	1,254
C_2	CH_3	CH_3CH_2	$C_{63}H_{88}N_{12}O_{16}$	1,268
C_3	CH_3CH_2	CH_3CH_2	$C_{64}H_{90}N_{12}O_{16}$	1,282

Table 1. Preparative resolution of the actinomycin C complex by CCC.

Fraction	Eluate volume (ml)	Weight (mg)	Composition or comment
1~4	132	8.9	Negligible 440 nm abs
5~8	264	22.3	Actinomycin C_3
9	297	5.3	Actinomycins C_2+C_3
10~12	396	18.9	Actinomycin C_2
13~14	462	2.0	Actinomycins C_1+C_2
15~18	594	2.3	Actinomycin C_1
19~22	726	0.5	Actinomycin C_1+more polar ones
Stationary phase		21.1	Complex mixture of polar materials

The separated layers were evaporated for weights and assayed for L1210 cytotoxicity. The system from equal parts of hexane - EtOAc - MeOH - H_2O approximately evenly distributed the weight as well as cytotoxicity of the sample and was used for CCC in the Ito multi-layer coil separator-extractor (P.C., Inc.). That system readily resolved a 100-mg sample of the complex from the broth extract into 29 mg of actinomycin C_3, 25 mg of actinomycin C_2, and 4 mg of actinomycin C_1 but required 1.7 liters of the upper phase which had been used as the mobile phase. Subsequent efforts, described below, led to an improved system which resolved the complex with a total volume of 600 ml of mobile phase.

Although a convenient HPLC procedure was available[4] for evaluating and quantitating the resolved actinomycins, each analytical determination required approximately 40 minutes, allowing analyses on only 12 samples in an 8-hour period. TLC densitometry offered the significant advantage of simultaneous analyses of multiple samples allowing considerable savings of time and money. Although bioautography or TLC densitometry on silica gel (Whatmann LK6DF) with methylethylketone - Me_2CO - H_2O (80:17:3 or 90:10:1), $CHCl_3$ - MeOH - H_2O (90:10:1), or on C18 (Whatmann LKC18D) with MeOH - H_2O (80:20 or 90:10) did not differentiate actinomycins C_2 and C_3 from an authentic sample of actinomycin D (C_1), a tenuous resolution of actinomycins C_1, C_2 and C_3 was achieved on silica gel with $CHCl_3$ - MeOH - H_2O (95:5:1) and provided the basis for a densitometric (Shimadzu CS-930) assay at 440 nm which facilitated monitoring CCC fractions. Multiple controls, mixed samples, and avoidance of overloads were helpful since the slight resolution was fragile. Although significant Rf variations have been observed, the relative Rf's of the components was constant. The following Rf ranges have been observed with this system; actinomycin C_1 (0.21~0.34), actinomycin C_2 (0.25~0.38), and actinomycin C_3 (0.28~0.42). Chromatographic identification of fractions was confirmed by fast atom bombardment MS (FAB-MS) and UV spectral analyses.

Armed with a convenient and rapid method for estimating actinomycin concentrations, we partitioned aliquots of the actinomycin C complex from the mycelial extract in a series of equilibrated biphasic solvent systems until we found that the system from ether - hexane - MeOH - H_2O (5:1:4:5) provided a roughly even distribution of the actinomycin components. That ethereal system was then used for CCC in the Ito coil with improved results. After loading the coil (i.d. 2.6 mm; volume about 385 ml) with lower phase (stationary), a solution of 83 mg of the actinomycin complex in a mixture of both phases was introduced through a loop valve (volume 9 ml) and mobile phase (upper) pumped through the coil rotated at 800 rpm. At a flow rate of 3 ml/minute, 358 ml or 93% of the stationary phase was retained. Collected fractions (33 ml each) of the mobile phase were evaporated for weights and analyzed by TLC densitometry. Results are summarized in Table 1.

Spectral analyses on the isolated actinomycins are summarized below. Actinomycin C_1 (D), UV λ_{max}^{MeOH} nm (ε) 238 (32,600), 426 (20,300) and 441 (21,300). FAB-MS $(M+H)^+$ at m/z 1,255. Actinomycin C_2, UV λ_{max}^{MeOH} nm (ε) 236 (sh, 38,800), 425 (24,300) and 442 (25,400). FAB-MS $(M+H)^+$ at m/z 1,269. Actinomycin C_3, UV λ_{max}^{MeOH} nm (ε) 236 (sh, 38,500), 425 (23,800) and 442 (24,600). FAB-MS $(M+H)^+$ at m/z 1,283.

CCC conveniently provided preparative sam-

ples of essentially pure actinomycins C_1, C_2 and C_3 with less than 600 ml of mobile phase. The constant elution profiles of these actinomycins in subsequent preparative resolutions has aided in their identification.

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