ISOLATION, PURIFICATION, SYNTHESIS, AND ANTIINVASIVE/ ANTIMETASTATIC ACTIVITY OF U-77863 AND U-77864 FROM *Streptomyces griseoluteus*, STRAIN WS6724

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In screening of actinomycetes for structures with differential solid tumor activity, *Streptomyces griseoluteus*, strain WS6724 was found to produce U-77863 and U-77864. U-77863 exhibited antiinvasive activity *in vitro* in the membrane invasion culture system (MICS) and a dose-dependent antimetastatic activity *in vivo* versus K1735-M2 and B16-F10 murine melanomas. The isolation, purification, and synthesis of both structures and biological activity is reported.

In the course of screening actinomycetes for agents with differential solid tumor activity, U-77863 and U-77864 were isolated from *Streptomyces griseoluteus*, strain WS6724. Chromatography of extracts of the clear beer and mycelia yielded two pools of eluate that were found to be active *in vitro* in the Wayne State University two-tumor soft agar assay.[1)]

The first pool of fractions, 6724A, yielded *trans*-3-(2′-methylphenyl)-2-propene-1-carboxamide (U-77863) (**I**, Fig. 1) and the second, 6724B, yielded 3-hydroxy-3-(2′-methylphenyl)-propane-1-carboxamide (U-77864) (**II**). U-77864 was found to be a novel structure while a structure corresponding to U-77863 was reported to have been synthesized.[2)] However, the analytical results reported in that paper did not match those obtained for U-77863. Synthesis was used to confirm both structures and to provide sufficient quantities of each for further biological studies.

We report the results of *in vitro* two-tumor assays of these compounds, as well as preliminary results of *in vivo* testing for toxicity and antitumor activity. Because of their relatively low toxicity and their structural characteristics, U-77863 and U-77864 were tested in the membrane invasion culture system (MICS), an *in vitro* model for measuring the ability of tumor cells to invade through reconstituted basement membrane matrices and these results are included here.[3)]

Results

Isolation, *In Vitro* Anti-tumor Activity, and Identification

Fermentations of the culture, WS-6724, were found to exhibit differential solid tumor activity *in vitro* (Table 1) and produced activity versus P388 leukemia *in vivo* (%T/C 155 at a 1:3 dilution of the whole beer, 1 ml injected ip, QID × 5, in BO1 mice). This culture was isolated from a soil sample of unknown origin. Comparisons of the Ektachrome photographs and electron micrographs of this strain with those of NRRL B-1315 and NRRL 3412 were made. This strain exhibited an aerial mass color of white to sparse grayish-pink, was melanin-negative, showed good growth at 28°C and no growth at 50°C, and gelatin

Fig. 1.

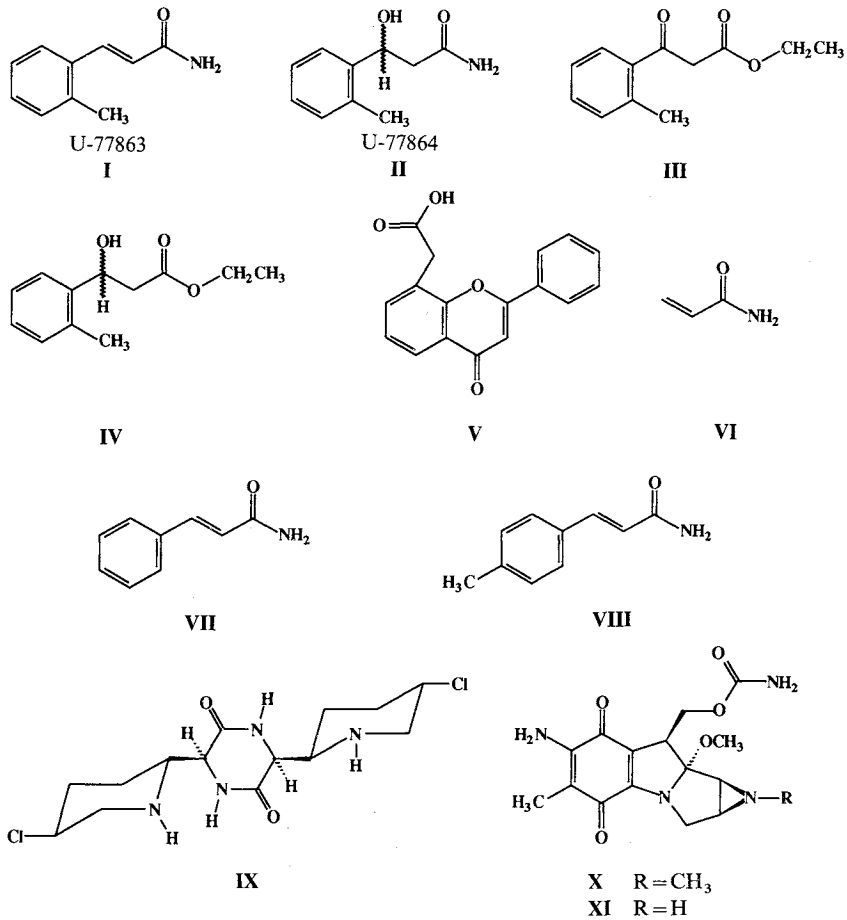
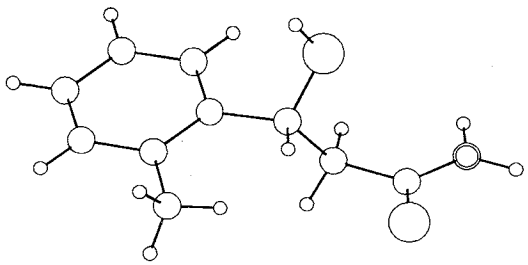


Table 1. Wayne State University two-tumor soft agar assay.

Sample	Concentration (mg/ml)	Dilution factor	Dose (μg/disk)	Zone diameter (μm)				
				Leukemias		Solid tumors		
				L1210	P388	C38	PO3	PO1
6724-WB (1st fermentation)	35	1×	—	0～116	—	—	320	—
6724-WB (2nd fermentation)	35	2×	—	—	0～540	—	>950	—
6724-CB	20	1×	—	—	330～370	—	780	—
n-BuOH ext/mycelia	10	1×	500	—	460	—	950	—
n-BuOH ext/6724-CB	10	1×	500	—	450	—	900	—
6724A	10	1/4×	125	—	370	—	480	—
6724B	10	1/10×	50	70	—	450	—	—
U-77863	10	1×	500	50	—	—	—	0
U-77864	10	1×	500	0	—	—	—	0
Acrylamide	10	1×	500	130	—	90	—	—
Cinnanamide	10	1×	500	—	—	—	—	0
4-Methylcinnanamide	10	1/2×	250	0	—	—	0	—
III	10	1×	500	0	—	—	0	—

Fig. 2.



U-77864

Table 2. Modified two-tumor assay.

Sample	Dose (mg/disk)	L1210 (units)	C38 (units)
U-77863	50	10	0
U-77864	50	70	>120
Flavone acetic acid	50	45	25

1 unit = 150 μm.

Table 3. L1210 tube dilution assay.

Sample	ID_{50} (μg/ml)	ID_{90} (μg/ml)
U-77863	>100	>100
U-77864	>100	>100
Acrylamide	58	>100
Cinnanamide	52	>100
4-Methylcinnamide	100	>100
Flavone acetic acid	>5	>5

liquefaction. From these studies, it was concluded that this culture is a strain identified as *Streptomyces griseoluteus*, strain WS6724.

Extraction of the clear beer at basic pH (8.0) and the mycelia with *n*-butyl alcohol after filtration in the presence of filter aid and subsequent evaporation of the extract recovered the majority of the activity present in the whole and clear beers of this fermentation (Table 1). Chromatography of the combined concentrated extracts on silica gel with a mobile phase of toluene-methyl isobutyl ketone (MIBK)-methyl alcohol yielded only two pools of fractions which upon evaporation showed activity in the two-tumor assay. The first, 6724A, showed essentially equal activity against leukemia and solid tumor cells while the second, 6724B, showed differential activity toward solid tumor cells.

The structures of the compounds isolated from pools 6724A and 6724B (U-77863 and U-77864, respectively) were determined by chemical, spectroscopic, optical rotation, and X-ray diffraction analyses. U-77863 was determined to be of the *trans* configuration by ^{1}H NMR spectroscopy due to the coupling constant (16 Hz) for the vinylic hydrogens.[4] As mentioned previously, a structure corresponding to that of U-77863 was reported, but the only data for the previously synthesized structure that was identified as 2'-methyl-3-phenylpropene-1-carboxamide was a melting point of 178°C.[3] Their reference to a reported melting point of 179°C for this structure,[5] was not supported by our inspection of that article. We have found the corrected melting point of 2'-methyl-3-phenylpropene-1-carboxamide to be 151~153°C. An optical rotation, $[\alpha]_D^{25} = 0°$, suggested that U-77864 consisted of a racemic mixture of the two stereoisomers generated by the chiral β-carbon. The X-ray diffraction spectra (Fig. 2) of U-77864 recrystallized from methyl alcohol confirmed that structure.

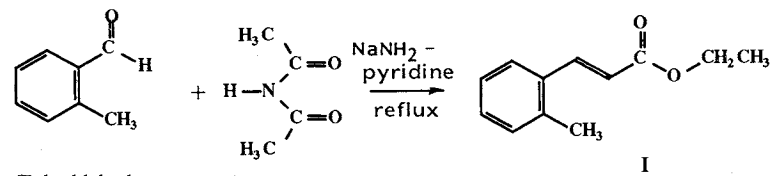
After recrystallization from MIBK, neither of the compounds isolated from the pools, 6724A and 6724B (U-77863 and U-77864, respectively) showed significant activity *in vitro* in the Wayne State University two-tumor assay, but did produce activity in a modified two-tumor assay (Table 2)[6] that was comparable to that shown for the evaporated pools, 6724A and 6724B. Both structures showed insignificant activity in the L1210 tube dilution assay (Table 3).[7] The supernatants from these recrystallizations did not produce any other active structures. We hypothesize that these compounds are more solubilized in the *in vitro* cells system of the modified two-tumor assay than in the Wayne State University two-tumor soft agar assay.

Synthesis of U-77863 and U-77864

A modified Perkin reaction involving *o*-tolualdehyde, acetamide, and potassium acetate produced

Fig. 3.

Scheme 1.

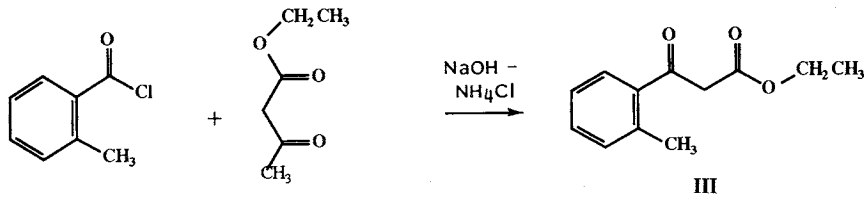


o-Tolualdehyde
C_8H_8O
FW = 120.15

Diacetamide
$C_4H_4NO_2$
FW = 101.11

I
U-77863
$C_{10}H_{11}NO$
FW = 161.20

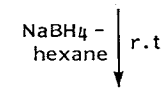
Scheme 2.

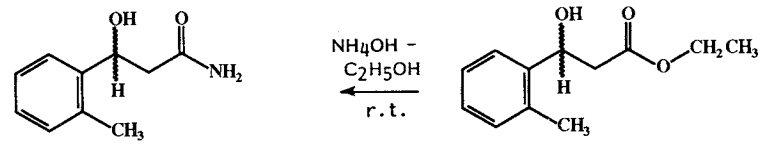


o-Toluoyl chloride
C_8H_7ClO
FW = 154.60

Ethyl acetoacetate
$C_6H_{10}O_3$
FW = 103.14

III
U-78720
$C_{12}H_{14}O_3$
FW = 206.24

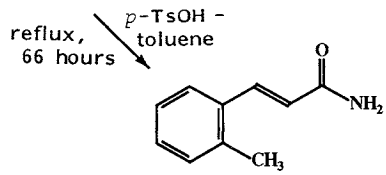




II
U-77864
$C_{12}H_{13}NO_2$
FW = 179.22

IV
U-89901
$C_{12}H_{16}O_3$
FW = 208.26

Scheme 3.



I
U-77863
$C_{10}H_{11}NO$
FW = 161.20

r.t.: Room temperature.

U-77863 in 27% yield (Scheme 1, Fig. 3).[8] The synthetic route to U-77864 (Scheme 2) involved the condensation of *o*-toluoyl chloride with ethyl acetoacetate in isopropyl alcohol-water (1 : 1) yielding **III** in 50% yield.[9] The β-ketoester was reduced with sodium borohydride to **IV** in 70% yield.[10] This β-hydroxy ester was then converted to U-77864 in 27% yield with excess NH_4OH in pyridine.[11] A sample of the synthesized U-77864 was then converted to U-77863 in 64% yield by refluxing for 66 hours with *p*-toluenesulfonic acid in toluene (Scheme 3).[12] Dehydration did not occur under milder conditions.

Spectroscopic (mass, NMR, IR and UV) and elemental analyses verified that the synthetic compounds were identical to those isolated from the beers.

In Vivo Antitumor Activity

Both structures were non-toxic (lethality) in mice at single doses $\leq$400 mg/kg (U-77863) and $\leq$800 mg/kg (U-77864) with ip administration. The higher non-lethal doses produced a central nervous system (CNS) stupor (*i.e.*, extremely low heart and respiratory rates and almost no response at all to reflex stimuli). Flavone acetic acid (FAA, NSC-347512), (**V**) was reported to have produced analogous results.[13] U-77863 and U-77864 were tested *in vivo* against P388 leukemia (Q4D $\times$ 3; ip tumor, ip drug), B16 melanoma (QID $\times$ 9; ip, ip), and Lewis lung carcinoma (QID $\times$ 9; ip, ip). Both U-77863 and U-77864 were inactive in those studies up to total doses of 400 and 800 mg/kg, respectively.

Invasion Assay

An obvious criteria for a true antiinvasive or antimetastatic effect was that the compounds should be active without killing tumor cells. Because of the low toxicity of U-77863 and U-77864 in the *in vitro* and *in vivo* antitumor assays, they were submitted for testing in the MICS. Toxicity was further evaluated *in vitro* in clonogenic colony formation assays. U-77863 powder was dissolved in DMSO at a concentration of 100$\sim$500 mg/ml immediately prior to each experiment. Drug was added to tissue culture medium or partially diluted in HANK's balanced salt solution before being added to cells. Final concentrations of DMSO were all less than any which cause effects on clonogenicity, growth, and/or invasion. Treatment of K1735-M2 and B16-F10 cells did not decrease clonogenicity at doses $\leq$200 μg/ml.

A dose-dependent inhibition of invasion was seen for both murine melanoma cell lines (Table 4). In the first experiments, 98% inhibition of invasiveness in the MICS was observed following pretreatment with 12.5 μg/ml U-77863 for 48 hours. This value varied from 42$\sim$90% inhibition in subsequent experiments with the average IC_{50} for both cell lines between 5 and 10 μg/ml with a 48-hour exposure. Similar experiments with human melanoma and rat mammary adenocarcinoma cell lines show that inhibition of invasion is greater if the cells are exposed for longer times.[14]

Results for other compounds that were initially tested using the same procedures as those used for U-77863 are also included in Table 4. U-77864 and FAA were inactive at the non-toxic doses selected. U-2422 (**VII**) and U-5414 (**VIII**) produced significant antiinvasive activity.

Table 4. Membrane invasion culture system assay.

Sample	Dose (μg/ml)	Inhibition (%)
U-77863	12.5	98
U-77864	50	0
Acrylamide	1.2	95
Cinnanamide	25	95
Flavone acetic acid	25	0

Table 5. Experimental metastasis formation by murine B16-F10 and K1735-M2 melanoma cells.

U-77863 (μg/ml)	Lung metastases		Extrapulmonary metastases
	Median No.	(Range)	Incidence
B16-F10			
Control	177	(1, >300)	10/15
0.625	137	(20, >300)	6/14
1.25	124	(32, 225)	9/15
2.50	114	(9, >300)	4/10
5.00	42	(7, 143)	2/15
10.0	99	(12, 214)	3/15
K1735-M2†			
Control	121$\pm$18		
1.00	116$\pm$13		
5.00	82$\pm$19*		
10.0	43$\pm$13*		

† 5×10^4 cells iv, 2 weeks.

* Statistically different ($P<0.05$) from control.

Metastasis Assay

Initial *in vitro* results indicated that U-77863 could inhibit a key component of the metastatic process, "invasion". Pretreatment of B16-F10 cells for 48-hour prior to tumor cell injection resulted in a slight dose-dependent inhibition of lung and extrapulmonary colonization in syngeneic C57BL/6 mice (Table 5). That there was any effect at all is remarkable since tumor had to grow, invade locally, and then complete the remaining steps in the metastatic process. Nonetheless, these experiments suggested that U-77863 had a relatively long-acting, statistically significant, inhibitory effect on the number of metastases under these stringent conditions. Interestingly, primary tumor growth was not affected by pretreatment with U-77863.

A more direct measure of effectiveness was assessed using the experimental metastasis assay and K1735 melanoma cells. Pretreatment for 48 hours with U-77863 resulted in a dose-dependent inhibition of the number of lung colonies formed in syngeneic C3H mice following intravenous injection of 5×10^4 cells (Table 5). Similar experiments with human melanoma cells revealed a dose-dependent, statistically significant inhibition as well. These *in vivo* and the previously described *in vivo* results have been confirmed in human and rat tumors, and potential mechanisms for the antiinvasive and antimetastatic activity of U-77863 are being studied.[14]

Experimental

General

Corrected melting points were obtained using the Mettler FP62 melting point apparatus and melting curves for standard materials. Infrared data was obtained using a DIGILAB Model FTS15E spectrophotometer. Nuclear magnetic resonance data, ^1H and ^{13}C, were obtained on a Bruker 200 MHz spectrometer. Elemental analyses were obtained using a Perkin-Elmer Model 240B elemental analyzer.

Isolation of U-77863 and U-77864

Filter aid (5% w/v) was added to the whole beer from fermentation culture, WS-6724, and the resulting suspension was filtered *in vacuo*. Extraction of the clear beer (4.0 liters) at natural pH (8.0) and the mycelia with *n*-butyl alcohol ($4 \times 2{,}000$ ml and $4 \times 1{,}000$ ml, respectively) and subsequent evaporation *in vacuo* of the combined extracts yielded 7.68 g of solid material. Chromatography of this solid material over 800 g of Silica Gel 60 (230~400 mesh) with toluene-methyl isobutyl ketone (MIBK)-methyl alcohol (65:25:10) as mobile phase was initiated. The initial 950 ml of eluate was discarded and 20 ml fractions were collected at a rate of 2 ml/minute. The individual fractions of eluate were combined into pools based upon TLC (Analtech Silica Gel GF, 250 plates, toluene-MIBK-methanol, 65:25:10) and visualization under UV illumination of representative fractions. After evaporation *in vacuo*, the first pool, fractions 76~93, yielded 0.29 g of white, apparently crystalline solid designated as 6724A. The second pool, fractions 112~183, yielded 0.49 g of a second, white, apparently crystalline solid designated as 6724B. Subsequent recrystallization of each of these solids from MIBK yielded 0.16 g of further purified solid from the 6724A pool and 0.26 g of further purified solids from the 6724B pool. Portions of each of these solids were analyzed chemically and spectroscopically and the structures of the solids from 6724A and 6724B were identified as U-77863 and U-77864, respectively. A portion of the solids from 6724B which had been recrystallized from MIBK was further recrystallized from methyl alcohol and submitted for X-ray diffraction spectroscopy. This further confirmed the structure of U-77864.

Tumor Cell Selection and Clonogenicity

The B16-F10 melanoma cell line, syngeneic to the C57BL/6 strain, was selected for high lung colonization and is widely used in the study of metastasis.[15] The K1735-M2 cell line is a clone selected from a lung metastasis from the K1735 melanoma in syngeneic C3H mice.[16]

U-77863 was added to tumor cells which had been plated at least one day previously. After treatment

for 48 hours, the medium was aspirated, the cells detached and plated as a single cell suspension onto 6-well tissue culture plates. Formation of colonies ($\geq$35 cells) was assessed 7 days after plating and compared to untreated, control cells. Treatment of K1735-M2 and B16-F10 cells did not decrease significantly ($\geq$10%) clonogenicity at doses $\leq$200 μg/ml.

MICS Invasion Assay

Invasion was quantified using the MICS with minor modifications. Using a 24-well manifold chamber fitted with a silicone gasket, a 10-μm pore polycarbonate filter coated with reconstituted basement membrane (Matrigel) was placed between the upper and lower chambers of MICS. A single cell suspension of K1735-M2 or B16-F10 cells was placed into the upper wells. After 72 hours, the cells were collected from the lower chamber using a 0.125% trypsin/2 mM EDTA solution in calcium- and magnesium-free DULBECCO's phosphate buffered saline. Invasion was determined by counting the cells after they had been separated from the liquid using a 3-μm pore polycarbonate filter. Data are reported as the number of invading cells per high power field.

Metastasis Assays

Metastasis can be measured in two ways. The first, called the spontaneous metastasis assay, involves injection of tumor cells into a site where a primary tumor will grow. Tumor cells must then invade from the primary tumor, enter the vasculature or lumphatics, travel to distant sites and form a secondary tumor. The second assay, called the experimental metastasis assay, bypasses the first steps of tumor growth to assesses the ability of blood-borne tumor cells to form colonies. In the experimental metastasis assay, tumor cells are injected directly into the vasculature. In the B16 and K1735 melanoma model systems, results are analogous. To evaluate the effectiveness of U-77863 on tumor growth and metastasis, B16-F10 melanoma cells were tested in the spontaneous metastasis assay. Lung colonization of K1735-M2 cells was evaluated in the experimental metastasis assay.

Murine melanoma cells were pretreated for 48 hours with U-77863. Subconfluent (70~90% confluence) K1735-M2 (a generous gift of Dr. I. J. FIDLER, University of Texas, M. D. Anderson Cancer Center) cells were detached by removal of medium followed by addition of 1 ml of 2 mM EDTA. When the cells began to retract, the plates were tapped to dislodge the cells. Ice-cold medium was added to dilute the EDTA. Cells were pelletted by centrifugation, the medium removed and the cells resuspended in ice-cold HANK's balanced salt solution (HBSS) at a concentration of 2.5×10^5 cells/ml. Cells (5×10^4) were injected into the lateral tail vein. Two weeks later, the mice were killed and the lungs removed. Surface lung colonies were counted as previously described.

Subconfluent B16-F10 cells were prepared as above except that they were suspended at a concentration of 5×10^6 cells/ml. Cells (1×10^6) were injected into the dorsolateral flank. After 25 days, the mice were killed and subjected to a complete gross necropsy. Surface lung metastases were counted and the incidence of extrapulmonary metastases recorded. Extrapulmonary metastases were observed in adrenal glands, diaphram, liver and ovary.

Synthesis of U-77863 (**I**)

To 500 ml pyridine (Mallinckrodt #7180) was added 33 g (840 mmol) $NaNH_2$ and 26.5 g (265 mmol) diacetamide (Aldrich #D5959, lot AP022226DK) followed by dropwise addition of 25 g (210 mmol) *o*-tolualdehyde (Aldrich #11,755-2 lot NM 02306 TJ). After refluxing for 20 hours, the mixture was stirred in 1,000 ml H_2O. This mixture was extracted with 3×500 ml $CHCl_3$ and the extract evaporated, yielding 14.6 g solid material. The solid was dissolved in 500 ml $CHCl_3$ and extracted with 3×250 ml 2.0 N NaOH. The residual $CHCl_3$ phase was evaporated, yielding 9.3 g (27%) of pure product; mp 151~153°C; TLC Rf 0.38, toluene - MIBK - CH_3OH (65 : 25 : 10); UV λ_{max}^{MeOH} nm (ε) 208 (17,320), 220 (14,900), 225 sh (12,030), 276 (16,540); IR ν_{max} (mineral oil) cm^{-1} 1668.3, 2925.0, 1610.5, 2953.8, 2854.5, 3380.1, 1635.5, 2869.0, 1399.2, 3185.3, 753.1, 627.7, 976.8, 726.0, 1485.0, 1459.0, 677.0, 971.0, 1379.0, 3292.3, 1447.5, 1249.7, 954.6, 1286.5, 1137.8; ^1H NMR ($CDCl_3$, TMS, δ) 2.435 (s, 3H); 5.868~6.361 (br, 2H); 6.383 (d, 1H, J=16 Hz); 7.160~7.261 (m, 3H); 7.514 (d, 1H); 7.933 (d, 1H, J=16 Hz); ^{13}C NMR ($CDCl_3$, TMS, δ) 168.467, 140.318, 137.580, 133.653, 130.791, 129.716, 126.213, 120.679, 19.842; EI-MS m/z 161.08 (Calcd for $C_{10}H_{11}NO$, 161.20).

Anal Calcd for $C_{10}H_{11}NO$: C 74.71, H 6.88, N 8.69.
Found: C 74.01, H 6.90, N 8.50.

Synthesis of **III**

To a stirred solution of 140 ml (1,290 mmol) ethyl acetoacetate (Aldrich #E964-1) in 2,000 ml of isopropyl alcohol - water (1 : 1) maintained at $<5°C$ in an ice bath, 288 ml (2,380 mmol) NaOH, 33% w/w, and 200 g (1,290 mmol) of *o*-toluoyl chloride (Aldrich #12,201-71) was added dropwise simultaneously from dropping funnels over 1 hour. The bath was removed and the contents heated to 35°C for 20 hours after which 129 g (2,400 mmol) NH_4Cl (Aldrich #21,333-0) was added. After an additional 24 hours stirring at 35°C, the mixture was concentrated to approximately 1 liter, and extracted with 3×500 ml hexane. The hexane extract was evaporated to yield 169 g (64%) of liquid product; Rf 0.50 (TLC, toluene - MIBK (75 : 25)); UV λ_{max}^{MeOH} nm (ε) 250 (19,830), 246 (9,750) 279 (3,330); IR ν_{max} (mineral oil) cm^{-1} 1742.5, 1688.5, 1193.8, 1260.3, 1202.5, 1319.2, 1300.0, 1289.3, 1034.7, 1626.8, 1149.5, 1641.3, 1410.8, 758.0, 1229.5, 1457.1, 1166.8, 770.5, 1382.8, 1367.5, 1601.7, 1136.0, 2482.0, 1446.5, 651.8; ^1H NMR ($CDCl_3$, TMS, δ) 1.242 (t, 3H); 2.459 (s, 1H); 2.520 (s, 1H); 3.914 (s, 2H); 4.155 (q, 2H); 7.115～7.495 (m, 3H); 7.633 (d, 1H); ^{13}C NMR ($CDCl_3$, TMS, δ) 195.558, 167.555, 139.264, 136.261, 132.441, 132.216, 129.225, 125.831, 61.220, 48.192, 21.467, 14.047; EI-MS *m/z* 206.09 (Calcd for $C_{12}H_{14}O_3$, 206.24).

Anal Calcd for $C_{12}H_{14}O_3$: C 69.89, H 6.84.
Found: C 70.18, H 6.86.

Synthesis of **IV**

To a stirred mixture of 62 g (1.63 mol) $NaBH_4$ (Alfa #14121) in 1,500 ml hexane, 169 g (815 mmol) U-78720 in 250 ml hexane was added dropwise over 15 minutes and the mixture was refluxed. After 22 hours, 2,000 ml water was added cautiously and stirring continued until all the solids had degraded. The phases were separated, the aqueous phase was extracted with $2 \times 1{,}000$ ml hexane, and the combined hexane extracts were evaporated, yielding 118 g (69%) of product; Rf 0.42 (TLC, toluene - MIBK (75 : 25)); $[\alpha]_D^{25}$ 0° (CH_3OH); UV λ_{max}^{MeOH} nm (ε) 245 (1,280), 271 (629), 290 (490); IR ν_{max} (neat) cm^{-1} 1734.0, 1182.2, 1162.0, 1266.1, 1035.6, 1203.5, 1300.0, 1371.2, 759.8, 615.1, 1315.3, 1064.6, 729.0, 1463.0, 2981.8, 1407.0, 3479.5, 1445.5, 1112.8, 1350.0, 1489.0, 2934.5, 2935.5, 1096.5, 1476.5; ^1H NMR ($CDCl_3$, TMS, δ) 1.190 (t, 3H); 2.272 (s, 3H); 2.564 (d, 2H); 3.693 (br, 1H); 4.089 (q, 2H); 5.254～5.297 (m, 1H); 7.038～7.174 (m, 3H); 7.403～7.440 (m, 1H); ^{13}C NMR ($CDCl_3$, TMS, δ) 172.399, 140.777, 134.234, 130.734, 127.458, 126.311, 125.292, 66.966, 60.787, 42.294, 18.919, 14.122; EI-MS, molecular ion detected (fragment ions assigned: 190 $C_{12}H_{15}O_2-H_1$; 145 $C_{10}H_{11}O_2-H_2O$; 115 $C_5H_9O_3-H_2$; 91 C_7H_7).

Anal Calcd for $C_{12}H_{16}O_3$: C 69.21, H 7.74.
Found: C 69.17, H 7.84.

Synthesis of U-77864 (**II**)

To 6.0 g (29 mmol) of U-89901 was added 20 ml (290 mmol) concentrated NH_4OH with stirring. Absolute ethanol (15 ml) was added to clarify the stirring mixture. After 17 hours stirring this mixture at room temperature, TLC (Analtech Silica Gel GF, 250 plates, toluene - MIBK - methanol, 65 : 25 : 10) showed U-77864 to be the major component. The mixture was evaporated, added to 200 ml CCl_4, stirred, and filtered. The filtered solids were extracted with 3×100 ml CH_3OH, filtered, and the filtrate was evaporated, yielding 2.6 g (49%) of pure product; mp 190～195°C; $[\alpha]_D^{25}$ 0° (CH_3OH); Rf 0.31 (TLC, toluene - MIBK - CH_3OH (65 : 25 : 10)); UV λ_{max}^{MeOH} nm (ε) 210 (10,440), 263 (467), 271 (428), 282 sh (213); IR ν_{max} (mineral oil) cm^{-1} 2918.3, 2954.9, 2854.6, 1675.2, 2868.1, 3351.3, 1461.1, 1455.3, 1057.0, 613.4, 1619.2, 3180.6, 766.7, 728.1, 641.3, 1378.1, 666.4, 1410.0, 1312.6, 698.2, 888.2, 1120.6, 1484.2, 1366.6, 1601.9; ^1H NMR (DMF, TMS, δ) 2.326 (s, 3H); 2.472 (s, 1H); 2.500 (d, 2H); 5.281～5.342 (m, 1H); 6.877 (br, 1H); 7.055～7.219 (m, 3H); 7.369 (br, 1H), 7.520 (s, 1H); ^{13}C NMR (DMF, TMS, δ) 174.066, 144.061, 134.870, 127.314, 126.481, 126.177, 67.697, 44.384, 18.911; EI-MS *m/z* 179.09 (Calcd for $C_{10}H_{13}NO_2$, 179.22).

Anal Calcd for $C_{10}H_{13}NO_2$: C 67.02, H 7.37, N 7.82.
Found: C 66.46, H 7.84, N 7.34.

Dehydration of U-77864 (**II**) to U-77863 (**I**)

To a 500 ml flask fitted with a water trap and nitrogen purge was added 0.5 g (2.8 mmol) U-77864, 0.54 g (0.28 mmol) *p*-toluenesulfonic acid, monohydrate (Aldrich #T3,592-0, FW = 190.22, 99%), and 250 ml of toluene and the mixture was stirred at room temperature for 6 hours with no reaction occurring. Subsequently, this mixture was stirred at reflux for 66 hours when TLC (Analtech Silica Gel GF, 250 plates, toluene - MIBK - methanol, 65 : 25 : 10) showed the reaction to be complete. The solvent was evaporated and the residue extracted with 3×250 ml $CHCl_3$. The extract was filtered and evaporated, yielding 0.287 g (64%) of the product.

Discussion

Neither U-77863 nor U-77864 produced significant differential solid tumor activity *in vivo*. However, the very significant *in vitro* antiinvasive activity and the *in vivo* antimetastatic activity of U-77863 and the apparent lack of activity for U-77864 suggest that both may be very important leads in the development of antiivasive and antimetastatic therapies. In addition, the induction of the CNS stupor in mice, analogous to FAA, as well as the low lethal toxicity may be a significant discovery. Correspondingly, taxonomic studies found that another strain of *Streptomyces griseoluteus* produced the piperazinedione, 593A, (**IX**),[17] and *Streptomyces ardus* which produced porfiromycin (**X**) and mitomycins A and C (**XI**), also produced cinnamic acid amide.[18] These observations, the previously unrecognized antiinvasive and antimetastatic activity, and the other activities described herein suggest that U-77863 and U-77864 may be involved in the biosynthetic pathways that produce some antitumor agents, are important leads in the development of antiinvasive/antimetastatic compounds, and may contribute to a better understanding of antitumor and CNS activity.

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