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Antitumor Agents. 25.[1] Synthesis and Antitumor Activity of Uracil and Thymine α-Methylene-γ-lactones and Related Derivatives

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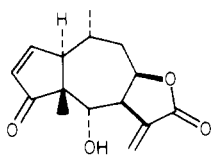
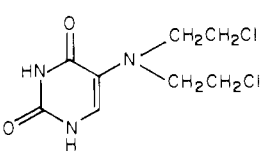
Uracil and thymine α-methylene-γ-lactones and related derivatives have been synthesized as novel potential alkylating antitumor agents. The synthesis of these compounds involved the convenient Reformatsky-type reaction between ethyl α-(bromomethyl)acrylate and the proper pyrimidinyl ketones. Preliminary in vivo tumor assay indicated that these compounds were active against the Walker 256 carcinosarcoma in rats and the P-388 lymphocytic leukemia as well as the B-16 melanotic melanoma in mice at 2.5–25 mg/kg.

Extensive screening of plant extracts has led to the isolation of a large number of sesquiterpene lactones having cytotoxic antitumor activity.[2-5] Our previous investigation of the structure–activity relationships for these compounds has indicated that one of the structural requirements for significant cytotoxic antitumor activity is an O=C—C=CH_2 system as part of an ester as well as a ketone (such as a β-unsubstituted cyclopentenone) or lactone (such as an α-methylene-γ-lactone).[3,6-9] This type of system has been observed, for example, in the cytotoxic antitumor helenalin (**1**).[6,7] It has been demonstrated that this O=C—C=CH_2 system could act as an alkylating center for the cytotoxic antitumor lactones and ketones, such as elephantopin,[10] vernolepin,[10] helenalin,[11] and tenulin.[11] A Michael-like reaction between biological nucleophiles, such as L-cysteine,[10,11] glutathione[11] or sulfhydryl-containing enzymes (e.g., phosphofructokinase,[12] glycogen synthetase,[13] DNA polymerase[11]), and –C=CH_2 grouping of the γ-lactone or the cyclopentenone moiety of these compounds has been proposed.

Our recent work on the synthesis of compounds derived from the combination of an above active alkylating center and a carrier moiety, such as a steroidal hormone, had led to several novel steroidal α-methylene-γ-lactone alkylating agents which are active against Walker 256 carcinosarcoma in rats.[14] These agents might be tumor specific (e.g., for breast or prostatic cancer) and thus clinically useful. As an extension of this approach we report herein the synthesis of α-methylene-γ-lactone with incorporation of nucleic acid base as a carrier moiety. The introduction of a carrier moiety into an alkylating center has led to compounds, such as uracil mustard (**2**), of clinical interest as anticancer agents.[15]

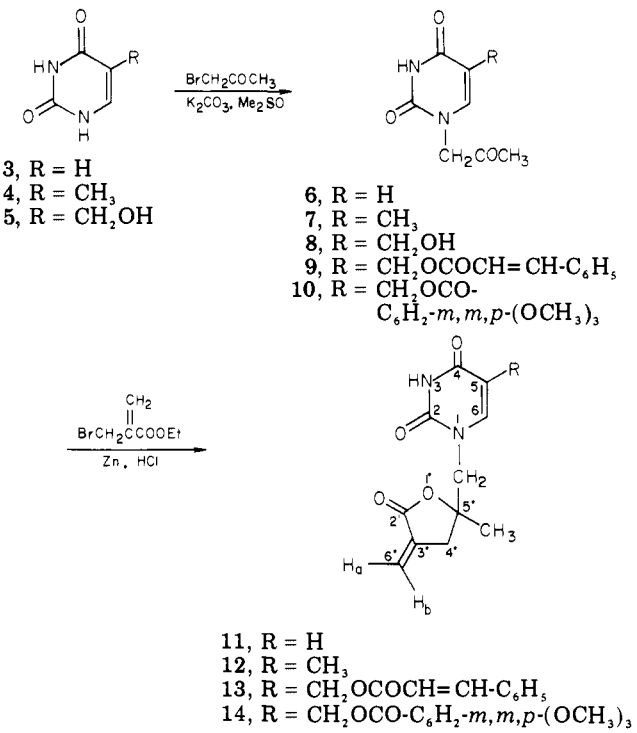


1



2

Scheme I



3, R = H
4, R = CH_3
5, R = CH_2OH

6, R = H
7, R = CH_3
8, R = CH_2OH
9, R = $CH_2OCOCH{=}CH\text{-}C_6H_5$
10, R = $CH_2OCO\text{-}C_6H_2\text{-}m,m,p\text{-}(OCH_3)_3$

11, R = H
12, R = CH_3
13, R = $CH_2OCOCH{=}CH\text{-}C_6H_5$
14, R = $CH_2OCO\text{-}C_6H_2\text{-}m,m,p\text{-}(OCH_3)_3$

Furthermore, a perusal of the literature revealed no record of any investigation on the synthetic α-methylene-γ-lactone bearing nucleic acid bases as alkylating anticancer agents except for the adeninyl- and uracilyl-furanones in which the double bond in the γ-lactone ring is either endocyclic or fully substituted.[16]

Chemistry. The synthesis of these uracil and thymine α-methylene-γ-lactones and their related derivatives (**11–14**) was carried out using the method similar to our previous work on the preparation of steroidal α-methylene-γ-lactones[14] and is outlined in Scheme I. The preparation of the α-methylene-γ-lactone moiety which

Table I. Effects of Uracil and Thymine α-Methylene-γ-lactones and Related Compounds on Inhibition of Tumor Growth

Compd	N[d]	Walker 256 ascites: Av days survived (2.5 mg/kg)	Walker 256 ascites: T/C[a]	P-388 lymphocytic leukemia: Av days survived (25 mg/kg)	P-388 lymphocytic leukemia: T/C	B-16 melanotic melanoma: Av days survived (25 mg/kg)	B-16 melanotic melanoma: T/C
11	6	10.13/7.75	131	11.50/10.00	115	12.20/13.51	111
12	6	12.70/10.00	127	12.70/10.00	127	27.00/22.00	123
13	6	23.80/11.30	211	12.30/9.50	130	27.60/19.60	141
14	6	23.70/11.30	209	13.50/9.50	142	25.30/19.60	129
15 (eupahyssopin)	6	26.40/8.00	330	14.60/9.90	147	20.80/18.80	111
16 (eupaformosanin)	6	36.50/13.40	471	14.60/9.90	147	21.20/18.80	113
Melphalan[b]	6	23.00/7.25	317				
5-Fluorouracil[c]	6			18.40/9.90	186	30.90/18.80	164

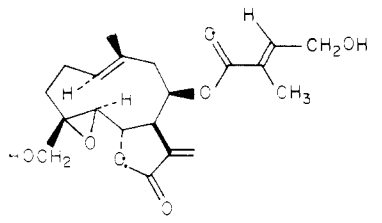
[a] A compound is active if it exhibits a T/C of ≥125%.[21] [b] Wellcome Research Laboratories, Research Triangle Park, N.C. [c] Calbiochem, La Jolla, Calif. [d] N is the number of animals per group.

involved a convenient Reformatsky-type reaction between ethyl α-(bromomethyl)acrylate[17] and a carbonyl group was originally reported by Öhler et al.[18] and has recently been applied to the synthesis of potential antitumor agents by Rosowsky et al.[19] as well as Howie et al.[20] The pyrimidinyl ketones **6–10** were obtained by condensation of the pyrimidines **3–5** with either bromoacetone or chloroacetone in dry Me_2SO in the presence of anhydrous K_2CO_3. Subsequent Reformatsky-type reactions led to the formation of desired products (**11–14**) whose compositions and spectral data were in accord with the assigned structures. 5-Hydroxymethyluracil-1-ylacetone (**8**) was esterified with cinnamoyl chloride and trimethoxybenzoyl chloride before being subjected to the Reformatsky-type reaction. The introduction of an additional conjugated ester side chain, such as cinnamoyl or trimethoxylbenzoyl moiety, into the 5-hydroxymethyl group of compounds **13** and **14** was due to the fact that such conjugated ester systems had been reported previously to enhance the cytotoxic antitumor activity of helenalin (**1**) and related derivatives.[7] In addition, the ester moiety protected the hydroxy group of **8** and thus prevented it from being poisoned by Reformatsky's reagent.

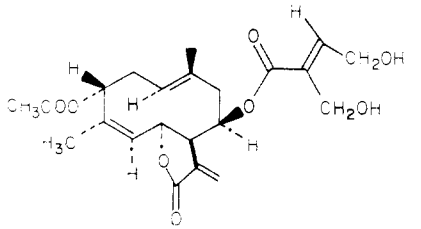
Biological Results. Compounds **3**, **4**, and **11–14** prepared in this study were assayed for their in vivo antitumor activity against the Walker 256 carcinosarcoma, the P-388 lymphocytic leukemia, and the B-16 melanotic melanoma according to standard NCI protocols.[21] As shown in Table I, compounds **12–14** exhibited significant activity in all of the above three types of tumors in low dose (2.5–25 mg/kg). The antitumor activity was enhanced with the introduction of the conjugated ester side chain (compare **13** and **14** to **11** and **12**) in the Walker 256 carcinosarcoma screen although no significant increase in activity was observed in the P-388 assay (Table I). The significant activity demonstrated by compounds **12**, **13**, and **14** in a low dose in the B-16 screen appears to be important since compounds **15** (eupahyssopin)[22] and **16** (eupaformosanin),[23] two naturally occurring germacranolide antitumor agents, which are highly active in the Walker 256 screen, were not active in the B-16 assay (Table I). Compounds **3** and **4** were inactive in all screens tested. Further investigation of the structure–activity relationships of the nucleic acid base α-methylene-γ-lactones and related derivatives is in progress.

Experimental Section

Chemistry. Unless otherwise specified, melting points were determined on a Thomas-Hoover melting point apparatus and were corrected. NMR spectra were measured with a Jeolco C-60 HL spectrometer (Me_4Si) and chemical shifts reported in δ (ppm) units: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; and the *J* values in hertz. Silica gel for preparative TLC refers to Merck silica gel GF-254; and silica gel for the TLC refers to Merck silica gel G developed with benzene–acetone (5:1) and visualized by spraying with sulfuric acid and heating. Elemental analyses were performed by M-H-W Laboratories, Garden City, Mich. Where analyses are indicated by only symbols of the elements, the analytical results for those elements were within ±0.4% of the theoretical value.



15



16

Uracil-1-ylacetone (6). To a solution of uracil (**3**, 1.8 g, 16 mmol) in dry Me_2SO (40 mL) was added 2.0 g (16 mmol) of anhydrous K_2CO_3. After the mixture was stirred at room temperature for 30 min, 1.2 mL (14 mmol) of bromoacetone in 14 mL of Me_2SO was added over a period of 4 h. The resulting solution was further stirred at room temperature for another 11 h and then filtered. Evaporation of the filtrate under reduced pressure gave a brownish semisolid. This was extracted with $CHCl_3$ and the $CHCl_3$ extract was washed with H_2O, dried (anhydrous Na_2SO_4), and evaporated in vacuo to yield a pale yellow solid. Recrystallization from $CHCl_3$–*n*-hexane with a small amount of Me_2SO afforded **6** as colorless needles (1.32 g, 56%): mp 209–210 °C; NMR (Me_2SO-d_6) 2.19 (3 H, s, CH_3), 4.66 (2 H, s, CH_2), 5.50 (1 H, d, *J* = 8.0 Hz, H-5), and 7.57 (1 H, d, $J_{5,6}$ = 8.0 Hz, H-6). Anal. ($C_7H_8N_2O_3$) C, H, N.

5′-Methyl-5′-(uracil-1-ylmethyl)-2′-oxo-3′-methylenetetrahydrofuran (11). To a solution of compound **6** (168 mg, 1 mmol) in dry THF (15 mL) was added activated zinc powder (200 mesh, 85 mg, 1.3 mmol) and *p*-hydroquinone (2 mg). To the mixture was added dropwise a solution of ethyl α-(bromomethyl)acrylate (260 mg, 1.3 mmol) in dry THF (5 mL) in 1 h. The reaction mixture was heated under reflux for 4 h. After cooling, it was poured into ice-cold 5% HCl (100 mL) and extracted with $CHCl_3$. The $CHCl_3$ extract was washed with H_2O, dried, and concentrated in vacuo to 10 mL, to which was then added dropwise *n*-hexane. The precipitate formed was filtered and recrystallized from $CHCl_3$–*n*-hexane to produce 150 mg (64%) of **11**: mp 200–201 °C; NMR (Me_2SO-d_6) 1.32 (3 H, s, CH_3-5′), 2.89 (2 H, m, CH_2-4′), 3.95, 3.98 (2 H, 2 s, NCH_2), 5.62 (1 H, overlapped d, $J_{5,6}$ = 8.0 Hz, H-5), 5.73 (1 H, overlapped t, $J_{b,4'}$

= 2.5 Hz, H_b), 6.07 (1 H, t, $J_{a,4'}$ = 3.0 Hz, H_a), and 7.58 (1 H, d, $J_{5,6}$ = 8.0 Hz, H-6). Anal. ($C_{11}H_{12}N_2O_4$) C, H, N.

Thymin-1-ylacetone (7). Thymine (**4**, 2.017 g, 16 mmol) was condensed with bromoacetone (1.918 g, 1.2 mL, 14 mmol) in the presence of K_2CO_3 (2.0 g, 14.5 mmol) and Me_2SO (56 mL) using the procedure similar to that for the preparation of **6**. The product was extracted with $CHCl_3$ from the brownish oily residue after the removal of Me_2SO. Repeated recrystallization from $CHCl_3$–*n*-hexane and a trace of Me_2SO gave **7** as colorless crystals (1.65 g, 65%): mp 197 °C; NMR (Me_2SO-d_6) 1.77 (3 H, s, CH_3-5), 2.33 (3 H, s, $COCH_3$), 4.60 (2 H, s, NCH_2), and 7.40 (1 H, s, H-6). Anal. ($C_8H_{10}N_2O_3$) C, H, N.

5′-Methyl-5′-(thymin-1-ylmethyl)-2′-oxo-3′-methylenetetrahydrofuran (12). To a mixture of compound **6** (516 mg, 3 mmol), activated zinc powder (195 mg, 3 mmol), and *p*-hydroquinone (6 mg) was added dropwise a solution of ethyl α-(bromomethyl)acrylate (586 mg, 3 mmol) in dry THF (15 mL). After the reaction mixture was heated under reflux for 10 h, another portion of ethyl α-(bromomethyl)acrylate (195 mg, 3 mmol) was added. The mixture was further refluxed for another 5 h. After cooling, it was filtered and worked up in the exact manner described for compound 11. After recrystallization from $CHCl_3$–*n*-hexane the product formed colorless needles (**12**, 675 mg, 90%): mp 210–212 °C; NMR (Me_2SO-d_6) 1.37 (3 H, s, CH_3-5′), 1.77 (3 H, s, CH_3-5), 2.90 (2 H, m, CH_2-4′), 3.93, 3.97 (2 H, 2 s, NCH_2), 5.70 (1 H, t, $J_{b,4'}$ = 3.0 Hz, H_b), 6.00 (1 H, t, $J_{a,4'}$ = 3.0 Hz, H_a), and 7.37 (1 H, br s, H-6). Anal. ($C_{12}H_{14}N_2O_4$) C, H, N.

5-Hydroxymethyluracil-1-ylacetone (8). A mixture of 5-hydroxymethyluracil (**5**, 2.548 g, 32 mmol), dry Me_2SO (40 mL), and anhydrous K_2CO_3 (3.980 g, 32 mmol) was allowed to react with a solution of chloroacetone (2.590 g, 2.24 mL, 28 mmol) in dry Me_2SO (15 mL) in an analogous manner as described for the conversion of **3** to **6**. The reaction mixture was filtered and the filtrate was concentrated under reduced pressure. The product was precipitated by addition of $CHCl_3$ to the concentrated solution. The precipitate was filtered, washed with 80 mL of MeOH, and then recrystallized twice from Me_2SO–MeOH to yield colorless crystals (**8**, 3.2 g, 57%): mp 217 °C; NMR (Me_2SO-d_6) 2.20 (3 H, s, $COCH_3$), 4.20 (2 H, s, CH_2OH), 4.70 (2 H, s, NCH_2), 7.43 (1 H, s, H-6). Anal. ($C_8H_{10}N_2O_4$) C, H, N.

5-Cinnamoyloxymethyluracil-1-ylacetone (9). A mixture of compound **8** (693 mg, 3.5 mmol) and cinnamoyl chloride (913 mg, 5.5 mmol) in 50 mL of dry benzene and 10 mL of dry pyridine was refluxed for 60 h. The reaction mixture was filtered to remove the pyridine hydrochloride salt, and the filtrate was diluted with benzene and cooled. The resulting crystals were collected by filtration and washed with benzene to yield 645 mg of crude compound **9**. The filtrate was further concentrated to small volume to which *n*-hexane was added. The precipitate thus formed was filtered and washed first with a mixture of $CHCl_3$–benzene (1:1) and then with benzene until the cinnamoyl chloride was completely removed as indicated by TLC [silica gel, acetone–benzene (1:5), triple development] to provide an additional 391 mg of crude compound **9**. The crude products were combined and recrystallized from $CHCl_3$–*n*-hexane to yield colorless needles (**9**, 1.036 g, 90%): mp 210 °C; NMR (acetone-d_6 with three drops of Me_2SO-d_6) 2.15 (3 H, s, $COCH_3$), 4.70 (2 H, s, NCH_2), 4.87 (2 H, s, CH_2O), 6.46 (1 H, d, J = 16.5 Hz, C*H*=CHPh), 7.70 (1 H, d, J = 16.5 Hz, CH=C*H*Ph), 7.30–7.80 (6 H, m, aromatic protons and H-6). Anal. ($C_{17}H_{16}N_2O_5$).

5′-Methyl-5′-[(5-cinnamoyloxymethyl)uracil-1-ylmethyl]-2′-oxo-3′-methylenetetrahydrofuran (13). A mixture of compound **9** (658 mg, 2 mmol), ethyl α-(bromomethyl)acrylate (1.04 g, 5.2 mmol), activated zinc powder (340 mg, 5.2 mmol), and a trace of *p*-hydroquinone (4 mg) in dry THF (60 mL) was refluxed overnight (18 h) and cooled. The reaction mixture was filtered and the filtrate was poured into ice-cold 5% HCl and extracted with $CHCl_3$. The $CHCl_3$ extract was washed with H_2O, dried, and evaporated to give yellowish viscous residue which was further purified by preparative TLC [silica gel, benzene–acetone (1:1)] followed by recrystallization from $CHCl_3$–*n*-hexane to give 710 mg (85%) of **13**: mp 149–150 °C; NMR [acetone-d_6–Me_2SO-d_6 (1:1)] 1.50 (3 H, s, CH_3), 2.87 (1 H, t, J = 3.0 Hz, H-4′), 3.00 (1 H, t, J = 3.0 Hz, H-4′), 3.85 (1 H, d, J_{gem} = 15 Hz, NCH_2), 4.20 (1 H, d, J_{gem} = 15 Hz, NCH_2), 5.00 (2 H, s, CH_2O), 5.69 (1 H, t, $J_{b,4'}$ = 3.0 Hz, H_b), 6.20 (1 H, t, $J_{a,4'}$ = 3.0 Hz, H_a), 6.47 (1 H, d, J = 16.5 Hz, C*H*=CHPh), 7.78 (1 H, d, J = 16.5 Hz, CH=C*H*Ph), and 7.30–7.70 (6 H, m, aromatic protons and H-6). Anal. ($C_{21}H_{20}N_2O_6$) C, H, N.

5-(*m*,*m*,*p*-Trimethoxybenzoyloxymethyl)uracil-1-ylacetone (10). A mixture of compound **8** (990 mg, 5 mmol), 3,4,5-trimethoxybenzoyl chloride (1.78 g, 7.7 mmol) in dry benzene (60 mL), and dry pyridine (10 mL) was refluxed until the reaction was complete as indicated by TLC. The reaction mixture was worked up in an analogous manner as described for the conversion of **8** to **9** to yield, after recrystallization from $CHCl_3$–*n*-hexane, colorless crystals (**10**, 1.57 g, 80%): mp 203 °C; NMR (Me_2SO-d_6) 2.20 (3 H, s, CH_3), 3.83 (3 H, s, *P*-OCH_3), 3.88 (6 H, s, *m*-OCH_3), 4.73 (2 H, s, NCH_2), 5.03 (2 H, s, CH_2O), 7.27 (2 H, s, aromatic protons), and 7.87 (1 H, s, H-6). Anal. ($C_{18}H_{20}N_2O_8$) C, H, N.

5′-Methyl-5′-[5-(*m*,*m*,*p*-trimethoxybenzoyloxymethyl)-uracil-1-ylmethyl]-2′-oxo-3′-methylenetetrahydrofuran (14). Compound **10** (510 mg, 1.3 mmol) was allowed to condense with ethyl α-(bromomethyl)acrylate (785 mg, 4 mmol) in the presence of 340 mg of activated zinc powder in dry THF (50 mL) according to the procedure described for the conversion of **9** to **13**. The product formed colorless crystals, after recrystallization from $CHCl_3$–*n*-hexane (**14**, 551 mg, 75%): mp 183–184 °C; NMR ($CDCl_3$) 1.50 (3 H, s, CH_3), 2.90 (2 H, m, H-4′), 3.90, 3.93 (9 H, 2 s, OCH_3), 5.15 (2 H, s, CH_2O), 5.70 (1 H, t, $J_{b,4'}$ = 3.0 Hz, H_b), 6.25 (1 H, t, $J_{a,4'}$ = 3.0 Hz, H_a), 7.40 (2 H, s, aromatic protons), and 7.73 (1 H, s, H-6). Anal. ($C_{22}H_{24}N_2O_9$) C, H, N.

In Vivo Tumor Screens. In the Walker 256 ascites carcinosarcoma screen, 10^6 tumor cells were implanted ip into Sprague–Dawley male rats (~80 g). Test compounds were administered ip (2.5 mg/kg/day). T/C values were calculated. Melphalan was used as a positive standard.

In the P-388 lymphocytic leukemia screen, 10^6 cells (hemocytomete) were implanted ip into male DBA/2 mice (~20 g) on day 0. Test compounds were administered ip at 25 mg/kg/day for 2 weeks. T/C values were calculated from average survival times. 5-Fluorouracil was used as a positive standard.

In the B-16 melanotic melanoma screen, 1 g of tumor was homogenized in 10 mL of Hanke's balanced salt solution and 0.5 mL of the homogenate was implanted sc in the auxillary region of the inguinal region of the leg of C_{57} Bl/6 male mice (~30 g). Test compounds were administered ip (25 mg/kg/day for 2 weeks). T/C values were calculated. 5-Fluorouracil was used as a positive standard.

T/C > 125 is considered significant for any of the screens. Test compounds were homogenized in 0.05% Tween 80.

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Microbial Transformations of Natural Antitumor Agents. 3. Conversion of Thalicarpine to (+)-Hernandalinol by *Streptomyces punipalus*

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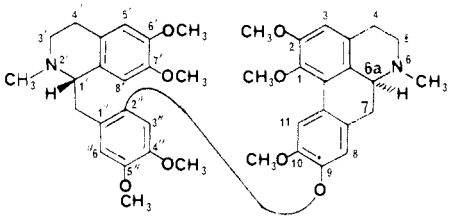
Microbial transformation studies were conducted with the antitumor alkaloid thalicarpine. *Streptomyces punipalus* (NRRL 3529) converted thalicarpine to (+)-hernandalinol, the structure of which was determined spectroscopically and by synthesis from the known alkaloid hernandaline. This unusual biotransformation reaction most likely occurs by oxidative cleavage of the isoquinoline ring from thalicarpine through the intermediate hernandaline, which then undergoes further reduction to hernandalinol.

Microbial transformation systems have been successfully utilized in the study of natural antitumor drug biotransformations.[1-4] We have been developing microbial transformations as a general means for (a) providing quantities of potentially active metabolites of complex antitumor compounds from nature; (b) preparing difficult to synthesize metabolites of such compounds which may be used as analytical standards to facilitate mammalian drug metabolism studies; and (c) determining potentially important pathways of bioactivation, bioinactivation, and cytotoxicity which may also occur in mammalian species. Previous reports from our laboratory concerned the microbial hydroxylation of acronycine,[1] an antitumor acridone alkaloid, and microbial N-dealkylation of *d*-tetrandrine,[2] a bis(benzyltetrahydroisoquinoline) alkaloid. This report describes the results of microbial transformation studies with the alkaloid thalicarpine (1a).



1a

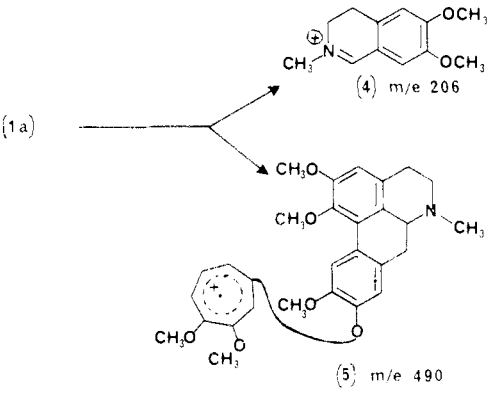
1b 6a,7-dehydro-

Thalicarpine, the first reported example of a benzyltetrahydroisoquinoline–aporphine dimeric alkaloid,[5] was originally isolated from *Thalictrum*[5,6] and *Hernandia*[7] species, and the correct structure for the alkaloid was shown to be 1a by total synthesis.[8] Thalicarpine has demonstrated significant antitumor activity against the Walker 256 carcinosarcoma[9] and cytotoxicity against monolayer cultures of KB cells.[10]

Results and Discussion

Of 22 cultures used in initial screening work those consistently yielding metabolites with thalicarpine were

Scheme I. Mass Spectral Fragments of Thalicarpine (1a)



(4) m/e 206

(5) m/e 490

Fusarium solani (ATCC 12823), *Streptomyces punipalus* (NRRL 3529), *Streptomyces griseus* (UI 1158), *Cunninghamella blakesleeana* (ATCC 8688a), and *Mucor mucedo* (UI 4605).

Since yields of metabolites were low, medium variation studies were performed in order to optimize the production of metabolites by these organisms. Each culture was grown in each of the media described under the Experimental section. Both the yields and numbers of metabolites were found to be highly dependent on the medium employed. *S. punipalus* gave relatively good yields of three metabolites on medium B containing glucose, maltose, or sucrose as the primary carbon and energy source. Since results with this organism were most reproducible, it was selected for a preparative scale fermentation.

A fermentation of *S. punipalus* containing 3.0 g of thalicarpine yielded 0.27 g of the major metabolite as well as two minor compounds after chromatography. Most of the thalicarpine added to the medium was recovered unchanged. The structure of the major metabolite was determined principally on the basis of NMR and mass spectral measurements and was confirmed by chemical synthesis.