Microbial Transformations of the Sesquiterpene Lactone Costunolide

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The microbial transformation of the sesquiterpene lactone costunolide has been studied. Preliminary screening experiments indicated a large number of fungi were capable of biotransforming costunolide. In preparative-scale fermentations, *Aspergillus niger* converted costunolide into dihydrocostunolide (5) and into four eudesmanolide derivatives, colartin (2), 11,13-dihydrosantamarine (6), 11,13-dihydroreynosin (7), and tetrahydrovulgarin (8) formed by a sequence in which costunolide was reduced, epoxidized, and then cyclized. *Cunninghamella echinulata* produced 1β-hydroxyarbusculin A (10) and *Fusarium oxysporum* produced dihydrocostunolide (5). A proposal of the sequence of events in the conversion of costunolide to the eudesmanolide metabolites is discussed.

THE biological activities of the sesquiterpene lactones have been reviewed,[1] and although they have been shown to possess a variety of biological activities, the current interest in these compounds lies mainly in their anticancer activity. A study of the structure–activity requirements of germacranolides concluded that an α-methylene-γ-lactone and αβ-unsaturated ester side-chain adjacent to the α-methylene-γ-lactone as well as allylic primary and/or secondary alcohols were required for high *in vivo* antitumour activity.[2]

Micro-organisms are capable of carrying out specific chemical transformations with a high degree of selectivity, as evidenced by the appearance of several catalogues documenting these conversions.[3-5] The microbial transformation of the germacranolide sesquiterpene lactone costunolide (1) was of particular interest since it is the most cytotoxic monofunctional sesquiterpene lactone which does not show *in vivo* activity,[6] and since allylic and aliphatic hydroxylations are common microbial conversions.[5] The resulting hydroxylated germacranolide sesquiterpenes or appropriately modified derivatives might be expected to exhibit *in vivo* antitumour activity.

RESULTS AND DISCUSSION

The costunolide (1) used in this study was obtained from *Magnolia grandiflora* as described,[7] and had physical and spectral data consistent with those reported in the literature.[7,8] Forty-five fungal cultures were screened on a small scale for their ability to biotransform costunolide. Although the rate and degree of conversion varied, forty organisms (Table) were capable of biotransforming costunolide to one or more metabolites. Three organisms, *Aspergillus niger* (ATCC 16888), *Cunninghamella echinulata* (NRRL 3655), and *Fusarium oxysporum* (ATCC 7601), were selected for preparative-scale study.

Four metabolites were isolated from preparative-scale fermentations using submerged cultures of *A. niger*. The first metabolite was optically active and had molecular formula $C_{15}H_{24}O_3$ (elemental analysis). The i.r. (KBr) spectrum and the ^1H n.m.r. ($CDCl_3$) spectrum differ significantly from that of costunolide.[7,8] The spectral data for this metabolite corresponds to the known sesquiterpene colartin (2) [9] which could arise by reduction of the 11,13-exocyclic double bond in costunolide (1), followed by transannular cyclization and hydration of the intermediate carbonium ion to yield (2). The stereochemistry for the C-11 methyl group was established by noting that it experiences an upfield shift of 0.28 p.p.m. in [$^2H_6$]benzene (δ 0.93) relative to deuteriochloroform (δ 1.21), consistent with a pseudo-equatorial (α) methyl group.[10] The C-4 epimer of (2) has also been reported,[11] and since both epimers have similar m.p. and an authentic sample of colartin was not available, additional evidence to confirm structure (2) of the metabolite was obtained. Dehydration of (2)

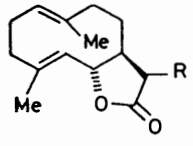
Cultures * yielding metabolites of Costunolide

Alternaria solani 11078
Aspergillus flavipes 1030
A. flavipes 11013
A. flavipes 16795
A. flavus 9170
A. flavus 24741
A. niger 10548
A. niger 11394
A. niger 16888
A. ochraceus 18500
A. ochraceus 22947
A. parasiticus 15517
Beauvaria bassiana 13144
Calonectria decora 14767
Chaetomium cochloides 10195
Cladosporium resinae 22712
Cunninghamella blakesleeana 8688a
C. echinulata 3655
C. echinulata 9244
C. echinulata 11585a
C. echinulata 11585b
C. elegans 9245
Curvularia lunata 12017
C. lunata 13633
Fusarium cepae 11711
F. javanicum 12575
F. gladioli 11137
F. oxysporum 7601
F. solani 11712
F. tulipae 15652
Mucor griseo-cyanus 1207
Penicillium adametzi 10407
P. chrysogenum 9480
P. chrysogenum 10002
P. frequentans 10444
P. oxalicum 24784
P. spinulosum 16348
Rhizopus arrhizus 11145
R. stolonifer 6227
Septomyxa affinis 6737
Syncephalastrum racemosum 18192

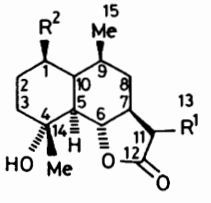
*All cultures were obtained from the American Type Culture Collection, Rockville, Maryland, except *Cunninghamella echinulata* 3655 which was obtained from the Northern Regional Research Laboratories, Peoria, Illinois.

with thionyl chloride in pyridine and separation on silver nitrate-impregnated silica gel G yielded α-cyclodihydrocostunolide (3) and β-cyclodihydrocostunolide (4). The dehydration products (3) and (4) had physical and spectral data consistent with those reported in the literature,[9] and their identities were confirmed by direct comparisons with authentic samples prepared by cyclization of dihydrocostunolide (5), according to the procedure described for cyclization of costunolide.[12] Additional supportive evidence for structure (2) comes by noting that methyl groups 1,3-diaxial to a hydroxy-group show a deshielding effect of 0.2—0.4 p.p.m. in pyridine relative to chloroform.[13] Since a deshielding effect for the C-10 methyl in (2) did not occur (δ 0.87, pyridine; δ 0.99, chloroform), a 1,3-diaxial relationship for the C-4 hydroxy-group and the C-10 methyl does not exist [as in the C-4 epimer of (2)], and thus the hydroxy-group must be equatorial as depicted in (2).

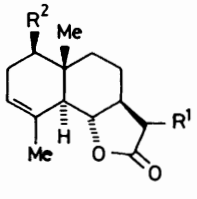
The second metabolite was optically active and had molecular formula $C_{15}H_{22}O_3$. The i.r. spectrum ($CHCl_3$) and the ^1H n.m.r. spectrum ($CDCl_3$) suggest that this metabolite is also a eudesmanolide derivative with a 3,4-endocyclic double bond as shown in (6), which would correspond to the known sesquiterpene 11,13-dihydrosantamarine (6). The metabolite had physical and spectral data consistent with those reported for 11,13-dihydrosantamarine [14,15] and its identity was confirmed by direct comparison with an authentic sample.



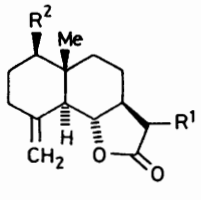
(1); R = =CH$_2$
(5); R = α—Me



(2); R^1 = α—Me; R^2 = H
(8); R^1 = α—Me; R^2 = OH
(10); R^1 = =CH$_2$; R^2 = OH
(15); R^1 = α—Me; R^2 = OCOMe
(16); R^1 = =CH$_2$; R^2 = H



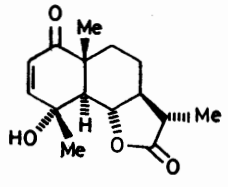
(3); R^1 = α—Me; R^2 = H
(6); R^1 = α—Me; R^2 = OH
(12); R^1 = =CH$_2$; R^2 = OH



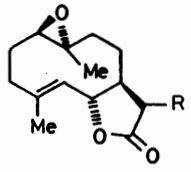
(4); R^1 = α—Me; R^2 = H
(7); R^1 = α—Me; R^2 = OH
(13); R^1 = =CH$_2$; R^2 = OH

The third metabolite ($C_{15}H_{22}O_3$) was also shown to be a crystalline optically active 11,13-dihydroeudesmanolide sesquiterpene lactone corresponding to the known sesquiterpene 11,13-dihydroreynosin (7),[16] and direct comparison with an authentic sample confirmed its identity.

The fourth crystalline metabolite ($C_{15}H_{24}O_5$) had i.r and ^1H n.m.r. spectral data consistent with an 11,13-dihydroeudesmanolide derivative. The metabolite formed a monoacetate which still showed hydroxy-bands in the i.r. spectrum as well as one D_2O-exchangeable signal in the ^1H n.m.r. spectrum, suggesting that it contained both secondary and tertiary hydroxy-groups. The fourth metabolite had physical and spectral data in agreement with those reported for tetrahydrovulgarin (8), a hydrogenation product of vulgarin (9).[17,18] Comparison of the metabolite with an authentic sample confirmed its identity.



(9)



(11); R = =CH$_2$
(14); R = α—Me

Preparative-scale fermentations using *Cunninghamella echinulata* resulted in the isolation of a single crystalline metabolite that was different from (2), (6), (7), and (8). The ^1H n.m.r. spectrum was similar to that of (8) except there was no methyl doublet; however, two one-proton doublets were evident. From the ^1H n.m.r. and i.r. spectral data the metabolite can be formulated as the structure shown in (10), which would correspond to the known sesquiterpene lactone 1β-hydroxyarbusculin A (10),[18,19] and the physical and spectral data as reported [18,19] are in agreement. The structure of the metabolite was confirmed by catalytic hydrogenation of (10) to give (8) which was identical in all respects to tetrahydrovulgarin (8).

1β-Hydroxyarbusculin A (10) was also prepared from 1,10-epoxycostunolide (11). The epoxide (11) was prepared as described in the literature [7] and then treated with 10% aqueous hydrochloric acid. The mixture that resulted was separated on silica gel G. In addition to the isomeric olefinic products santamarine (12) and reynosin (13), (10) was obtained and was identical in all respects to the metabolite obtained from *C. echinulata*.

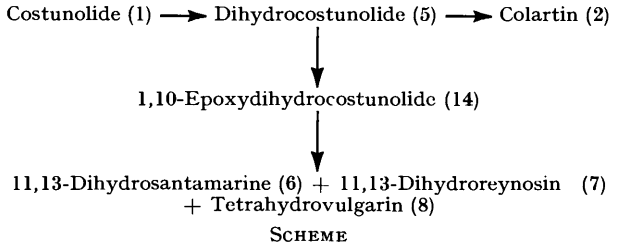
The third organism chosen for preparative-scale biotransformation, *Fusarium oxysporum*, was capable of biotransforming costunolide into one major crystalline metabolite. The ^1H n.m.r. is characteristic of germacranolides and is similar to that of costunolide except for the absence of the low-field doublets (H-13) and the presence of an additional three-proton doublet which suggests that the metabolite is dihydrocostunolide (5). The physical and spectral data are in agreement with those reported for dihydrocostunolide.[20,21] An authentic sample was prepared from costunolide by sodium borohydride reduction and was found to be identical in all respects with the metabolite.

Dihydrocostunolide (5) was also obtained as the major metabolite when costunolide was subjected to

biotransformation using stirred cultures of *A. niger*. Metabolites (2), (6), (7), and (8) were also produced but in much lower yield than in the shaken-culture fermentation studies with *A. niger*. The amounts and kinds of metabolites often differ between shaken and stirred cultures.

In all the biotransformation experiments, control studies were conducted in which costunolide was added to sterilized 24-h old submerged cultures. Under these conditions costunolide remained unchanged with no detectable (t.l.c.) decomposition products. It is known that costunolide can be cyclized with acidic reagents to give eudesmanolide products.[12] The pH of the fermentations was never below 4 and costunolide was stable under these conditions.

In a study of the microbial transformation of germacrone it was found that *Cunninghamella blakesleeana* stereoselectively epoxidizes germacrone to three epoxides. Acid-catalysed transannular cyclization of the 1,10-epoxide produced the corresponding 1β-hydroxyeudesmane derivatives.[22] 1,10-Epoxycostunolide (11) is also known to be very unstable and readily undergoes



SCHEME

cyclization.[7] Thus, a proposed explanation that would account for the production of metabolites from costunolide (1) by *A. niger* is shown in the Scheme. It is resonable to assume that the reduction takes place first since the corresponding α,β-unsaturated-γ-lactone derivatives [(10), (12), (13), and (16)] were not detected. These would result from acid-catalysed cyclization of 1,10-epoxycostunolide (11), or costunolide (1). Since 1,10-epoxydihydrocostunolide (14) could not be isolated from the fermentations, its proposed intermediacy was established by control studies in which (14) * was added to growing cultures of *A. niger* and metabolites (6), (7), and (8) were formed. Also, 1,10-epoxydihydrocostunolide (14) was added to sterilized cultures of *A. niger* and metabolites (6), (7), and (8) were obtained as well. The fact that (14) did produce (6), (7), and (8) when fed to sterilized cultures of *A. niger* (culture broth, pH 4) would suggest that acid-catalysed cyclization of (14), as well as enzyme-catalysed cyclization, may be operable.† However, when dihydrocostunolide (5) was fed to sterilized cultures of *A. niger*, there was no evidence (t.l.c.) of the formation of (2) after 7 d incubation, thus suggesting enzyme-mediated cyclization in this case.‡

Since only (10) was obtained when costunolide (1) was biotransformed by *C. echinulata* (culture broth, pH 6—7) this would indicate that cyclization to the eudesmanolide occurs enzymatically rather than from the acidity of the fermentation broth, particularly in view of the fact that when 1,10-epoxycostunolide was fed to sterilized cultures of *C. echinulata*, (10), (12), and (13) were formed, the same products as obtained on acid-catalysed cyclization using aqueous hydrochloric acid.

It is apparent from these biotransformation studies of costunolide with *A. niger*, *C. echinulata*, and *F. oxysporum* that three types of modifications have been achieved which include reduction of the α,β-unsaturated-γ-lactone function, epoxidation, and cyclization to eudesmanolide sesquiterpenes. All the metabolites [(2), (5)—(8), and (10)] were tested for cytotoxicity against KB cells [24] and as expected only (10) was active, since it is the only one containing the α,β-unsaturated-γ-lactone moiety necessary for cytoxicity.[6]

The desired hydroxylated germacranolides were not produced by *A. niger*, *C. echinulata* or *F. oxysporum*, nor was there any evidence that any of the metabolites detected from the other organisms listed in the Table were hydroxylated germacranolides.

EXPERIMENTAL

Physical data were obtained as follows: m.p. (uncorrected) determined on either a Thomas-Hoover Unimelt capillary melting-point apparatus or a Fisher-Digital model 355 melting-point apparatus; i.r. spectra, Beckman IR-33 or Perkin-Elmer 257 spectrophotometer; ^{1}H n.m.r. spectra, JEOL FX60 Fourier-transform n.m.r. spectrometer, $SiMe_4$ as internal standard; c.d. spectra, Jasco J-40 recording spectropolarimeter; optical rotations, Perkin-Elmer model 141 polarimeter (1 dm cell); low-resolution mass spectra, Dupont 21-492 mass spectrometer; chemical-ionization mass spectra determined by Battelle Memorial Laboratories, Columbus, Ohio; microanalyses performed by Scandinavian Microanalytical Laboratories, Herlev, Denmark.

T.l.c. was performed on pre-coated silica gel G-25 UV_{254} plates (0.25 mm). Preparative-layer chromatography was carried out on pre-coated silica gel plates (0.5-mm Sil G-50 UV_{254} or 2.0-mm Sil G-200 UV_{254}). The adsorbents used for column chromatography were silica gel 70—270 mesh, silica gel G for thin-layer chromatography, or silver nitrate-impregnated silica gel G. Silica gel G (for t.l.c.) was prepared for use in column chromatography by suspending 50 g of the silica gel G (t.l.c.) in 100 ml of water. The mixture was dried at 100 °C for 6 h and passed through a 60—80 mesh sieve. Silver nitrate-impregnated silica gel G for column chromatography was prepared by suspending 50 g of the silica gel G (t.l.c.) in 100 ml of an aqueous solution of silver nitrate (7.5 g). The mixture was dried at 100 °C for 6 h, passed through a 60—80 mesh sieve, and

* 1,10-Epoxydihydrocostunolide (14) was prepared from dihydrocostunolide (5) using the same procedure as described for the preparation of 11.[7] (14) has been prepared previously but only the m.p. was reported.[23] The formation of the expected 1,10-monoepoxide from (5) was established from ^{1}H n.m.r. data.

† The epoxide (14) was detected (t.l.c.) in the fermentation broth 8 h after the addition of costunolide (1) to 24-h old stage II cultures of *A. niger*. Metabolites (5), (6), (7), and (8) were also detected but not (2).

‡ Metabolite (2) can be detected (t.l.c.) in the fermentation broth 48 h after the addition of costunolide (1) to growing cultures of *A. niger*.

stored in amber-coloured jars. The t.l.c. plates were visualized by spraying with 20% H_2SO_4 in diethyl ether, followed by heating for 1—2 min.

Fermentation Procedures.—Stock cultures of fungi were stored on mycophil, potato-dextrose-yeast extract [potato dextrose agar + 2% extract] and Czapeks (Difco) agar slants at 4 °C. All cultures were purchased from the American Type Culture Collection, Rockville, Maryland, with the exception of *Cunninghamella echinulata* 3655, which was obtained from Northern Regional Research Laboratories, Peoria, Illinois. Biotransformation studies were carried out on gyrotory shakers (model G-10, New Brunswick Co.) operating at 200 revolutions min^{-1} and 26 °C in Erlenmeyer flasks holding one-fifth of their volume of a medium consisting of (per litre of H_2O): dextrose, 20 g; yeast extract, 5 g; peptone, 5 g; NaCl, 5 g; and K_2HPO_4, 5 g. Media were sterilized at 121 °C for 20 min. A two-stage fermentation procedure was utilized in which sterile liquid culture medium was inoculated with surface growth from agar slants. These stage I cultures were incubated as described for 48 h. The stage I culture broth (2 ml) was used as inoculum for stage II cultures. The substrate was added to 24-h old stage II cultures as a 5% ethanolic solution at a concentration of 0.1 mg ml^{-1} of stage II medium.

Culture controls consisted of fermentation blanks, in which the organisms were grown under identical conditions, including the addition of absolute ethanol, but without the substrate. Substrate controls consisted on the addition of the same amount of substrate to sterile media.

Fermentation Sampling.—Substrate-containing stage II cultures were sampled at intervals by aseptically withdrawing 5 ml of the culture broth. The samples were extracted with chloroform, evaporated to dryness, and the residues were spotted on t.l.c. plates which were developed in 8% EtOH–$CHCl_3$.

Preparative-scale Fermentation of Costunolide (1) *using* A. niger.—*Aspergillus niger* (ATCC 16888) was grown in 7.5 l of medium in 500-ml Erlenmeyer flasks. A total of 750 mg of costunolide (1) was distributed evenly among the cultures and the substrate-containing cultures were sampled daily, beginning 24 h after addition of the substrate. T.l.c. analysis of the samples taken indicated that the biotransformation was complete after 10 d incubation. The cultures were harvested by filtration and the aqueous culture broth (pH 4) was extracted with 8 l $CHCl_3$ (in 500-ml portions). The combined $CHCl_3$ layers were dried over Na_2SO_4 and evaporated *in vacuo* (40 °C) to leave a brown oily residue. The residue (1.78 g) was chromatographed over silica gel (70—235 mesh, 3.0 × 50 cm) using $CHCl_3$, followed by 0.5, 1.0, and 2.0% EtOH–$CHCl_3$ as eluant.

After elution of the column with 2.7 l of $CHCl_3$, 20-ml fractions were collected and combined on the basis of t.l.c. analysis.

Characterization of colartin (2). Continued elution of the column with $CHCl_3$ yielded (2) in fractions 3—12 (33 mg) which crystallized from ether–hexane, m.p. 108—110 °C (lit.,[9] 107—108 °C) (Found: C, 71.1; H, 9.4. $C_{15}H_{24}O_3$ requires C, 71.39; H, 9.59%); $[\alpha]_D^{25}$ +16.2° (*c* 0.16, $CHCl_3$); $\nu_{max.}$ (KBr) 3 610 (OH) and 1 785 (OCO) cm^{-1}; δ ($CDCl_3$) 0.99 (3 H, s, 10-Me), 1.21 (3 H, d, *J* 6.0 Hz, 11-Me), 1.31 (3 H, s, 4-Me), 3.02 (1 H, s, exchanges with D_2O, 4-OH), and 4.00 (1 H, dd, *J* 9 and 11 Hz, H-6); δ (C_6D_6) 0.55 (3 H, s, 10-Me), 0.93 (3 H, d, *J* 6.0 Hz, 11-Me), 1.19 (3 H, s, 4-Me), 3.24 (1 H, exchanges with D_2O, 4-OH), and 3.3 (1 H, m, H-6); δ (C_5D_5N) 0.87 (3 H, s, 10-Me), 1.15 (3 H, d, *J* 6.6 Hz, 11-Me), 1.34 (3 H, s, 4-Me), 4.16 (1 H, dd, *J* 8.5 Hz, H-6).

Characterization of 11,13-*dihydrosantamarine* (6). Elution of the column with 0.5% EtOH–$CHCl_3$ (800 ml) yielded fractions 13—58 (148 mg), a mixture of (2) and (6), which was further purified by preparative-layer chromatography (2.0-mm silica gel G) using 8% EtOH–$CHCl_3$ as the developing solvent. Extraction of the higher R_F band yielded an additional 83 mg of (2). Extraction of the lower R_F band afforded 49 mg of crude (6) which was further purified by preparative-layer chromatography (0.5-mm silica gel G, Et_2O). Extraction of the major band afforded 36 mg of a residue which was crystallized from benzene to give (6), m.p. 128—130 °C (lit.,[14] 124—125 °C; lit.,[15] 135.5—137 °C); $[\alpha]_D^{25}$ +56.2° (*c* 0.85, $CHCl_3$); $\nu_{max.}$ (KBr) 3 525 (OH) and 1 752 (OCO) cm^{-1}; $\nu_{max.}$ ($CHCl_3$) 3 614, 3 500 (OH), and 1 767 (OCO) cm^{-1}; δ ($CDCl_3$) 0.89 (3 H, s, 10-Me), 1.21 (3 H, d, *J* 6.5 Hz, 11-Me), 1.52 (exchanges with D_2O, CHO*H*), 1.80 (3 H, br s, 4-Me), 3.60 (1 H, dd, *J* 5.5 and 10.5 Hz, H-1), 3.88 (1 H, dd, *J* 11 and 11 Hz, H-6), and 5.27 (1 H, br s, H-3).

Direct comparison of (6) with an authentic sample of 11,13-dihydrosantamarine (6) showed no depression of melting point upon admixture, superimposable i.r. (KBr) spectra, and the same mobility on t.l.c.

Characterization of 11,13-*dihydroreynosin* (7). Continued elution of the column with 0.5% EtOH–$CHCl_3$ yielded fractions 68—81 (29 mg), which were pooled and shown by t.l.c. to consist primarily of (7). Further purification of the fraction by preparative-layer chromatography (0.5-mm silica gel G, Et_2O) afforded (7) from hexane, m.p. 128—129 °C (lit.,[16] 129 °C); $[\alpha]_D^{25}$ +121.8° (*c* 0.5, $CHCl_3$); $\nu_{max.}$ (KBr) 3 490 (OH), 1 755 (OCO), 1 653 (C=CH_2), and 885 (C=CH_2) cm^{-1}; $\nu_{max.}$ ($CHCl_3$) 3 615, 3 500 (OH), 1 767 (OCO), 1 672 (C=CH_2), and 872 (C=CH_2) cm^{-1}; δ ($CDCl_3$) 0.83 (3 H, s, 10-Me), 1.22 (3 H, d, *J* 6.5 Hz, 11-Me), 1.52 (exchanges with D_2O, CHO*H*), 3.48 (1 H, dd, *J* 5 and 11 Hz, H-1), 4.00 (1 H, dd, *J* 9 and 10.5 Hz, H-6), 4.78 (1 H, br s, H-14), and 4.92 (1 H, br s, H-14); δ (C_6D_6) 0.61 (3 H, s, 10-Me), 0.99 (3 H, d, *J* 6.3 Hz, 11-Me), 2.95 (1 H, dd, *J* 6 and 10 Hz, H-1), 3.4 (1 H, m, H-6), 4.93 (1 H, br s, H-14), and 5.22 (1 H, br s, H-14).

Direct comparison of (7) with an authentic sample of 11,13-dihydroreynosin (7) showed no depression of melting point upon admixture, the same mobility on t.l.c., and superimposable i.r. (KBr) spectra.

Characterization of tetrahydrovulgarin (8). Elution of the column with 2% EtOH–$CHCl_3$ yielded fractions 197—260 containing (8) (236 mg). The fraction was treated with a small volume of ether and the ether was decanted to leave a white solid (89 mg). Crystallization from benzene–ether afforded (8) (76 mg) as needles, m.p. 181—183° (lit.,[17,18] m.p. 197—199 °C) * (Found: C, 67.1; H, 8.95. $C_{15}H_{24}O_4$ requires C, 67.12; H, 9.02%); $[\alpha]_D^{25}$ +3.7° (*c* 0.99, C_6D_6); $\nu_{max.}$ ($CHCl_3$) † 3 585, 3 455 (OH), and 1 781 (OCO) cm^{-1}; δ ($CDCl_3$) 0.99 (3 H, s, 10-Me), 1.22 (3 H, d, *J* 6.5 Hz,

* The melting point of (8) increases to 196—198 °C when the crystals are ground into a fine powder. The m.p. of synthetic (8) is also 181—183 °C and upon grinding increases to 196—198 °C.

† The i.r. spectrum of (8) in KBr exhibits absorption bands at $\nu_{max.}$ 3 544, 3 423 (OH), 1 782, and 1 761 (OCO) cm^{-1}. Synthetic tetrahydrovulgarin, also exhibits two carbonyl absorption bands in its i.r. (KBr) spectrum.

11-Me), 1.34 (3 H, s, 4-Me), 3.10 (exchanges with D_2O, OH), 3.4 (1 H, m, H-1), and 4.08 (1 H, dd, *J* 9 and 9 Hz, H-6); δ (C_5D_5N) 1.17 (3 H, s, 10-Me), 1.18 (3 H, d, *J* 6.8 Hz, 11-Me), 1.44 (3 H, s, 4-Me), 3.6 (1 H, m, H-1), and 4.3 (1 H, H-6).

Direct comparison of (8) with an authentic sample of tetrahydrovulgarin (8) showed no depression in melting point upon admixture, the same mobility on t.l.c., and superimposable i.r. (KBr) spectra.

Characterization of Dihydrocostunolide (5).—Costunolide (1) (500 mg) was added to a 24-h old stage II culture of *Aspergillus niger* 16888 grown in 5 l of medium held in a 7.5-l fermentor jar. The culture was incubated (200 revolutions min^{-1}, 2 l min^{-1} air-flow rate) for 9 d. The aqueous culture broth was extracted with $CHCl_3$ (10 × 500 ml). The combined $CHCl_3$ layers were dried over Na_2SO_4 and evaporated *in vacuo* (40 °C) to leave a brown oily residue (863 mg). The residue was applied to a column of silica gel G (75 g, 1.9 cm × 32 cm) and the column was eluted with $CHCl_3$, followed by 1% EtOH–$CHCl_3$. Fractions of 10 ml were collected and pooled on the basis of t.l.c. analysis.

Elution of the column with $CHCl_3$ (330 ml) afforded a crystalline residue in fractions 33—50 (86 mg) from which dihydrocostunolide (5) (18 mg) was obtained as needles from light petroleum, m.p. 75—77 °C (lit.,[20,21] 77—78 °C).

Direct comparison of this metabolite with an authentic sample of dihydrocostunolide (5) showed no depression of mixed melting point, the same mobility on t.l.c., and superimposable i.r. (KBr) spectra.

Preparative-scale Fermentation of Costunolide (1) *using* Cunninghamella echinulata.—*Cunninghamella echinulata* 3655 was grown in 6.8 l of medium in 500-ml Erlenmeyer flasks. Costunolide (1) (680 mg) was evenly distributed among the cultures and the substrate-containing cultures were incubated as described for 3 d. The aqueous culture broth (pH 7) was extracted with $CHCl_3$ (8 × 1 000 ml) and the combined $CHCl_3$ layers were dried over Na_2SO_4 and evaporated *in vacuo* (40 °C) to leave a brown oily residue (1.386 g). The residue was dissolved in $CHCl_3$ (10 ml) and 1 ml of the solution was applied to each of ten preparative t.l.c. plates (2.0-mm silica gel G). The plates were developed in 8% EtOH–$CHCl_3$ and extraction of the major band yielded a residue (134 mg) which was further purified by preparative-layer chromatography (0.5-mm silica gel G, 8% EtOH–$CHCl_3$). Extraction of the major band afforded (10) as needles from benzene–ether, m.p. 192—194 °C (lit.,[18,19] m.p. 194—196 °C); $[\alpha]_D^{25}$ +17.3° (*c* 0.075, MeOH); $\nu_{max.}$ ($CHCl_3$) * 3 595, 3 460 (OH), 1 775 (OCO), and 1 660 (C=CH_2) cm^{-1}; δ ($CDCl_3$) 0.98 (3 H, s, 10-Me), 1.36 (3 H, s, 4-Me), 2.18 (exchanges with D_2O, OH), 3.4 (1 H, m, H-1), 4.13 (1 H, dd, *J* 11 and 11 Hz), 5.44 (1 H, d, *J* 3.0 Hz, H-13), and 6.12 (1 H, d, *J* 3.0 Hz, H-13); c.d. (MeOH) $[\theta]_{254}$ −2 821°, $[\theta]_{208}$ +15 357° {lit.,[19] c.d. (MeOH) $[\theta]_{254}$ −2 410°}.

Preparative-scale Fermentation of Costunolide (1) *using* Fusarium oxysporum.—*Fusarium oxysporum* 7601 was grown in 1.0 l medium in 500-ml Erlenmeyer flasks. Costunolide (1) (100 mg) was evenly distributed among the cultures and the substrate-containing cultures were incubated for 3 d. The aqueous culture broth (pH 7) was extracted with $CHCl_3$ (4 × 500 ml) and the combined $CHCl_3$ layers were dried over Na_2SO_4 and evaporated *in vacuo* (40 °C) to leave an oily residue (90 mg). The residue was purified by preparative-layer chromatography (0.5-mm silica gel G, 8% EtOH–$CHCl_3$). Extraction of the major band yielded a colourless oil (36 mg) which upon crystallization from light petroleum afforded (5) as needles, m.p. 75—76 °C (lit.,[20,21] 77—78 °C). Direct comparison of this metabolite with an authentic sample of dihydrocostunolide (5) showed no depression of mixed melting point, the same mobility on t.l.c., and superimposable i.r. (KBr) and ^1H n.m.r. spectra.

Control Studies.—Costunolide (90 mg) was evenly distributed among each of three 24-h old stage II cultures of *Aspergillus niger* 16888, *Cunninghamella echinulata* 3655, and *Fusarium oxysporum* 7601, which had been sterilized by autoclaving at 121 °C for 20 min. After a 4-d incubation period for all the cultures, there was no evidence (t.l.c.) of the formation of metabolites (2), (5)—(8), or (10), as well as any other products, from costunolide [silica gel G, 8% EtOH–$CHCl_3$; (1), R_F 0.74; (2), R_F 0.52; (5), R_F 0.63; (6) and (7), R_F 0.27; (8), R_F 0.25; (10), R_F 0.25].

Dihydrocostunolide (5) (30 mg) was evenly distributed among three 24-h old stage II cultures or *A. niger* 16888 which had been sterilized by autoclaving (121 °C, 20 min). After a 7-d incubation period, there was no evidence (t.l.c.) of the formation of metabolites (2), (6), (7), or (8), or any other products, from dihydrocostunolide [silica gel G, 8% EtOH–$CHCl_3$; (5), R_F 0.70; (2), R_F 0.60; 2% EtOH–$CHCl_3$; (5), R_F 0.45, (2), R_F 0.21].

1,10-Epoxycostunolide (11) (30 mg) was evenly distributed among three 24-h old stage II cultures of *C. echinulata* 3655 which had been sterilized by autoclaving. After a 24-h incubation period, t.l.c. analysis showed that all the epoxide (11) had undergone cyclization to three products corresponding in R_F value to santamarine (12), reynosin (13) and 1β-hydroxyarbusculin A (10) [silica gel G, 8% EtOH–$CHCl_3$; (10), R_F 0.29; (12) and (13), R_F 0.45; 15% $AgNO_3$-silica gel G, Et_2O; (12), R_F 0.54; (13), R_F 0.29].

1,10-Epoxydihydrocostunolide (14) (190 mg) was evenly distributed among nineteen 24-h old stage II cultures of *A. niger* 16888 which had been sterilized by autoclaving. After a 24-h incubation period, the aqueous culture broth (pH 4) was extracted with $CHCl_3$ (4 × 500 ml). The combined $CHCl_3$ layers were dried over Na_2SO_4 and evaporated *in vacuo* (40 °C) to leave a brown oily residue (186 mg). The residue was dissolved in $CHCl_3$ (2 ml) and applied equally to two 2.0-mm silica gel G t.l.c. plates, which were developed in 8% EtOH–$CHCl_3$. Extraction of the lower R_F band gave a residue (65 mg) which upon crystallization (benzene–ether) afforded (8), m.p. 181—183 °C (m.p. 196—198° as a powder), identical in all respects [m.p., mixed m.p.; t.l.c., joint t.l.c., superimposable i.r. (KBr) spectra] with (8) obtained from the biotransformation of costunolide by *A. niger* 16888.

Extraction of the higher R_F band yielded an oily residue (77 mg) which was shown by t.l.c. (15% $AgNO_3$–silica gel G, Et_2O) to be a mixture of two components. The residue was applied to a column of 15% $AgNO_3$–silica gel G (7 gm, 1 × 23 cm). The column was eluted with Et_2O and 3-ml fractions were collected. Fractions 4—6 were pooled and crystallized to give (6), m.p. 124—125 °C, identical in all respects [m.p., t.l.c., joint t.l.c., superimposable i.r. (KBr) spectra] with (6) obtained from the biotransformation of

* The i.r. spectrum of metabolite (10) in KBr exhibits $\nu_{max.}$ 3 570, 3 400 (OH), 1 785, and 1 750 (OCO) cm^{-1}, with the carbonyl bands (1 785 and 1 750 cm^{-1}) varying in intensity between different samples. Synthetic 1β-hydroxyarbusculin A (10), prepared from 1,10-epoxycostunolide (11), exhibits the same type of behaviour.

costunolide (1) by *A. niger* 16888. Crystallization of fractions 11—15 afforded (7), m.p. 128—130 °C, identical in all respects [m.p., mixed m.p., t.l.c., joint t.l.c., superimposable i.r. (KBr) spectra] with (7) obtained from the biotransformation of costunolide by *A. niger* 16888.

Dehydration of Colartin (2) *to* (3) *and* (4).—To a cooled solution (5 °C) of (2) (48 mg) in dry pyridine (2 ml) was added $SOCl_2$ (0.2 ml). After 10 min, the reaction mixture was diluted with H_2O and extracted with $CHCl_3$. The $CHCl_3$ layer was dried over Na_2SO_4 and evaporated *in vacuo* (40 °C) to leave an oily residue (19 mg). Preparative-layer chromatography [15% $AgNO_3$–silica gel G, light petroleum–isopropyl ether (3 : 1)] afforded crude (3) (12 mg). Crystallization from light petroleum gave (3) as clear plates, m.p. 128—130 °C (lit.,[9] 132—135 °C). Direct comparison of (3) with an authentic sample of α-cyclodihydrocostunolide (3) showed the two products to be identical [m.p., mixed m.p., t.l.c., joint t.l.c., superimposable i.r. (KBr) spectra].

Extraction of the lower R_F band gave crude (4) (12 mg). Crystallization from light petroleum afforded (4) as clear plates, m.p. 131—133 °C (lit.,[9] 138.5—140 °C). Direct comparison of (4) with an authentic sample of β-cyclodihydrocostunolide (4) showed no depression of mixed melting point, the same mobility on t.l.c., and superimposable i.r. (KBr) spectra.

Acetylation of (8).—Metabolite (8) was acetylated with acetic anhydride in pyridine to give the monoacetate (15) as needles (from ether–hexane), m.p. 135—136 °C (Found: C, 66.0; H, 8.55. $C_{17}H_{26}O_5$ requires C, 65.80; H, 8.39%), $[\alpha]_D^{25}$ +11.8° (*c* 0.15, $CHCl_3$); $\nu_{max.}$ (KBr) 3 605, 3 430 (OH), 2 935, 1 786 (OCO), 1 747 (OCOMe), and 1 237 (OCO) cm^{-1}; δ ($CDCl_3$) 1.10 (3 H, s, 10-Me), 1.25 (3 H, d, *J* 6.5 Hz, 11-Me), 1.42 (3 H, s, 4-Me), 2.10 (3 H, s, COMe), 3.03 (1 H, br s, exchanges with D_2O), 4.08 (1 H, dd, *J* 9 and 11 Hz, H-6), and 4.7 (1 H, m, H-1).

1,10-*Epoxydihydrocostunolide* (14).—Dihydrocostunolide (5) (75 mg) was dissolved in CH_2Cl_2 (20 ml) and stirred vigorously with 0.5M $NaHCO_3$ (20 ml). *m*-Chloroperbenzoic acid (72 mg) was added, in small portions, to the stirred solution and the mixture was stirred at room temperature for 1 h. The CH_2Cl_2 layer was separated, washed successively with 1.0M Na_2SO_3 (20 ml), 1.0M NaOH (40 ml), and H_2O (20 ml), dried over Na_2SO_4, and evaporated *in vacuo* (40 °C) to leave a crystalline residue (73 mg). Crystallization from alcohol–hexane afforded (14) as white needles, m.p. 136—138 °C (lit.,[23] 124—125 °C) (Found: C, 71.7; H, 8.75. $C_{15}H_{22}O_3$ requires C, 71.95; H, 8.86%); $[\alpha]_D^{25}$ +6.17° (*c* 0.115, C_6D_6); $\nu_{max.}$ (KBr) 2 935, 1 764 (OCO), and 1 670 (C=C) cm^{-1}; δ ($CDCl_3$) 1.13 (3 H, s, 10-Me), 1.23 (3 H, d, *J* 6.5 Hz, 11-Me), 1.81 (3 H, d, *J* 1.0 Hz, 4-Me), 4.55 (1 H, dd, *J* 11 and 11 Hz, H-6), and 5.17 (1 H, br d, *J* 10 Hz, H-5).

Reduction of 1β-*Hydroxyarbusculin* (10).—1β-Hydroxyarbusculin (10) (50 mg) was added to a suspension of 5% Pd–C (25 mg) in absolute ethanol (20 ml) which had been saturated with H_2. After the uptake of H_2 had ceased, the suspension was filtered through Celite 545 and the filtrate was evaporated *in vacuo* (40 °C) to leave a crystalline residue (44 mg). Crystallization from benzene–ether afforded (8), m.p. 195—197 °C, identical in all respects with an authentic sample of tetrahydrovulgarin (8) [m.p., mixed m.p., t.l.c., joint t.l.c., superimposable i.r. (KBr) spectra].

Cyclization of 1,10-*Epoxycostunolide* (11).—1,10-Epoxycostunolide (11) (300 mg) was stirred in 10% aqueous HCl for 30 min. The aqueous acidic suspension was extracted with CH_2Cl_2, and the CH_2Cl_2 layer was dried over Na_2SO_4 and evaporated *in vacuo* (40 °C) to leave a residue (295 mg) which was purified by preparative-layer chromatography (silica gel G, 8% EtOH–$CHCl_3$). Extraction of the lower R_F band gave a crystalline residue (80 mg) which, upon crystallization from benzene–ether, afforded (10) (24 mg) as needles, m.p. 193—195°, identical in all respects with (10) obtained from the biotransformation of costunolide by *C. echinulata* 3655 {m.p., mixed m.p., t.l.c., joint t.l.c., $[\alpha]_D$, c.d., superimposable i.r. (KBr), ^1H n.m.r. spectra}.

Reference Compounds.—An authentic sample of dihydrocostunolide (5), m.p. 75—77 °C (lit.,[20,21] 77—78 °C), was prepared by sodium borohydride reduction of costunolide (1) following the procedure used for the reduction of epitulipinolide.[25] 11,13-Dihydrosantamarine (6) was prepared by sodium borohydride reduction of santamarine (12), m.p. 124—125 °C (lit.,[14] 124—125 °C; lit.,[15] 135.5—137 °C). Tetrahydrovulgarin (8) was prepared by hydrogenation of vulgarin (9) using PtO_2 catalyst (HOAc, 30 lbf in^{-2}, 2 h), m.p. 181—183 °C (196—198 °C as a powder) (lit.,[17] 197—199 °C; lit.,[18] 197 °C). 1,10-Epoxycostunolide (11) was prepared according to the described procedure,[7] m.p. 125—126 °C (lit.,[7] 123—125 °C).

We acknowledge gifts of costunolide, vulgarin, and santamarine from Dr. F. S. El-Feraly, The University of Mississippi, and an authentic sample of 11,13-dihydroreynosin, kindly provided by Dr. Geoffrey A. Cordell, University of Illinois at the Medical Center. A. M. C. gratefully acknowledges support through an American Foundation for Pharmaceutical Education Fellowship. This work was supported in part by the Research Institute of Pharmaceutical Sciences, School of Pharmacy, and the Committee on Faculty Research, The University of Mississippi.

[8/2219 *Received*, 29*th December*, 1978]

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