Studies on the Acetogenins of Formosan Annonaceous Plants, II. Cytotoxic Acetogenins from Annona reticulata

Fang-Rong Chan, Yang-Chang Wu, Chang-Yih Duh, and Shang-Kwei Wang

J. Nat. Prod., **1993**, 56 (10), 1688-1694• DOI:
10.1021/np50100a005 • Publication Date (Web): 01 July 2004

Journal of Natural Products
Vol. 56, No. 10, pp. 1688-1694, October 1993

STUDIES ON THE ACETOGENINS OF FORMOSAN ANNONACEOUS PLANTS, II.[1] CYTOTOXIC ACETOGENINS FROM *ANNONA RETICULATA*

FANG-RONG CHANG, YANG-CHANG WU*, CHANG-YIH DUH,

Graduate Institute of Natural Products

and SHANG-KWEI WANG

Department of Microbiology, Kaohsiung Medical College, Kaohsiung 807, Taiwan, Republic of China

ABSTRACT—Using cytotoxicity as a guide to fractionation, one novel acetogenin, annoreticuin-9-one [**3**], and four known cytotoxic acetogenins, squamone [**4**], solamin [**5**], annomonicin [**6**], and rolliniastatin 2 [**7**], were isolated from active extracts of the leaves of the Formosan plant *Annona reticulata*. Their structures were elucidated on the basis of uv, ir, ^{1}H-and ^{13}C-nmr, and ms data of the natural compounds and their derivatives.

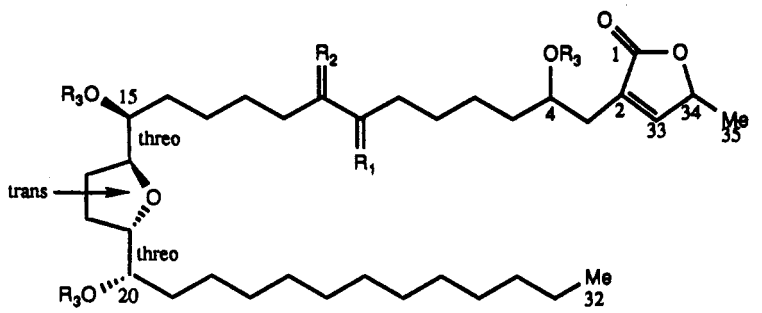
In a previous communication (1), we reported two new cytotoxic acetogenins, annoreticuin [**1**] and isoannoreticuin [**2**], from the leaves of Formosan grown *Annona reticulata* L. (Annonaceae). These two acetogenins possess a C_{35} skeleton with one tetrahydrofuran ring, one lactone moiety, and several hydroxyl groups. Further studies on the active extracts of the leaves have led to the isolation of one novel cytotoxic acetogenin, annoreticuin-9-one [**3**], as well as four known cytotoxic acetogenins, squamone [**4**], solamin [**5**], annomonicin [**6**], and rolliniastatin 2 [**7**]. These compounds have shown significant cytotoxicity and are currently under evaluation as potential anticancer agents.

RESULTS AND DISCUSSION

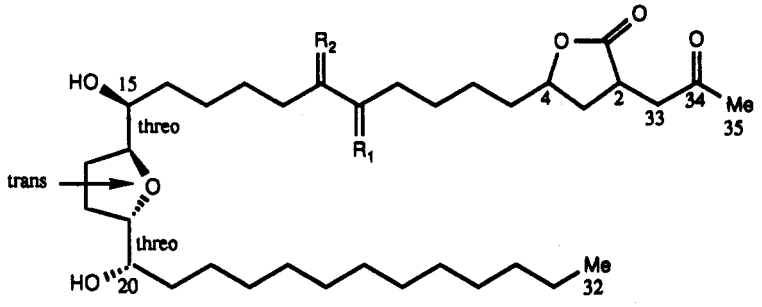
The MeOH extract of the leaves of *A. reticulata* was highly cytotoxic to KB (human nasopharyngeal carcinoma), A-549 (human lung carcinoma), and HCT-8 (human colon tumor) cell lines, as well as to murine P-388 and L-1210 lymphocytic leukemias. Using the P-388 system as a guide, the crude extract was fractionated by solvent partitioning, cc on Si gel and active carbon, and preparative tlc on Si gel, leading to the novel acetogenin annoreticuin-9-one [**3**], and the four known acetogenins squamone [**4**], solamin [**5**], annomonicin [**6**], and rolliniastatin 2 [**7**]. These pure compounds were shown to be cytotoxic in several systems and then characterized.

Annoreticuin-9-one [**3**] was obtained as a white amorphous powder, $[\alpha]^{25}D$ +11.7° (c=0.02, $CHCl_3$). The hrfabms gave an $[MH]^+$ ion at m/z 595.4535 (calcd 595.4557), consistent with a molecular formula of $C_{35}H_{62}O_7$. The presence of OH moieties was obvious by the loss of H_2O (m/z 18) from the molecular ion in the fabms and a broad absorption in the ir spectrum at 3430 cm^{-1}. The existence of three hydroxyls in contrast to annoreticuin [**1**], which had a formula of $C_{35}H_{64}O_7$ and four hydroxyls (1), was further confirmed by the formation of a triacetyl and a tris-trimethylsilyl (TMSi) derivative. A prominent ir carbonyl absorption at 1750 cm^{-1} and a uv λ max at 215 nm (log ε 3.4) suggested the presence of the α,β-unsaturated γ-lactone. Expected resonances in the ^{1}H-nmr and ^{13}C-nmr spectra (Table 1) confirmed the presence of an α,β-unsaturated γ-lactone as well as the presence of an OH group at C-4 (1,2). Subsequent ^{1}H-^{1}H decoupling experiments showed coupling between the protons on C-3, H_a-3 and H_b-3, to the single proton at C-4, establishing the presence of an OH group at C-4 as in

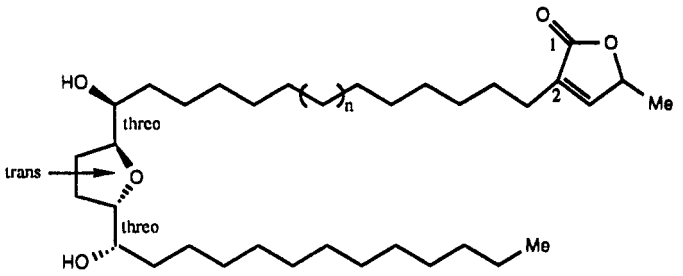
[1]For the previous paper in this series, see Wu *et al.* (1).



1 R_1=H,OH; R_2=H,H; R_3=H
3 R_1=O; R_2=H,H; R_3=H
triacetyl **3** R_1=O; R_2=H,H; R_3=Ac
tris-trimethylsilyl **3** R_1=O; R_2=H,H; R_3=TMSi
8 R_1=H,H; R_2=H, OH; R_3=H
10 R_1=H,H; R_2=O; R_3=H



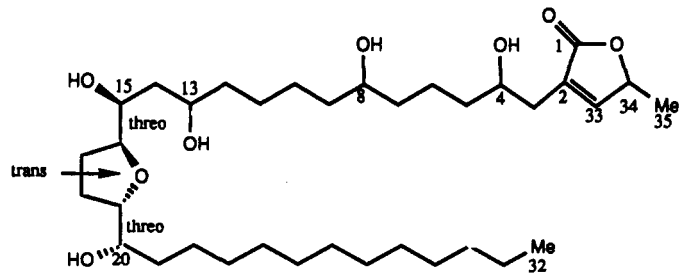
2 R_1=H,OH; R_2=H,H
4 R_1=O; R_2=H,H
9 R_1=H,H; R_2=H,OH
11 R_1=H,H; R_2=O



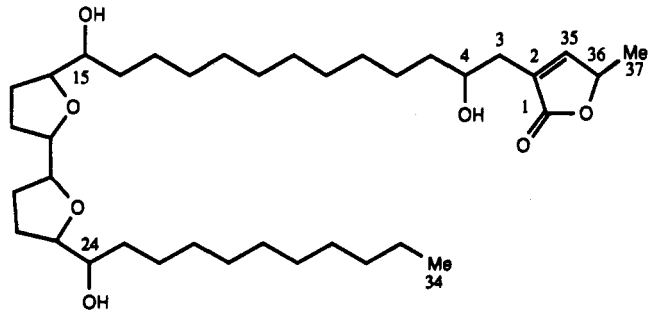
5 *n*=1
12 *n*=3

annonacin (3), goniothalamicin (4), and annoreticuin (1). The other spectral properties of **3** and **1** were very similar. Typical resonances in the ^{1}H-nmr spectrum at δ 7.21 (d, 1.4 Hz), 5.08 (qd, 6.8 and 1.4 Hz), and 1.44 (d, 6.8 Hz) supported this assignment.

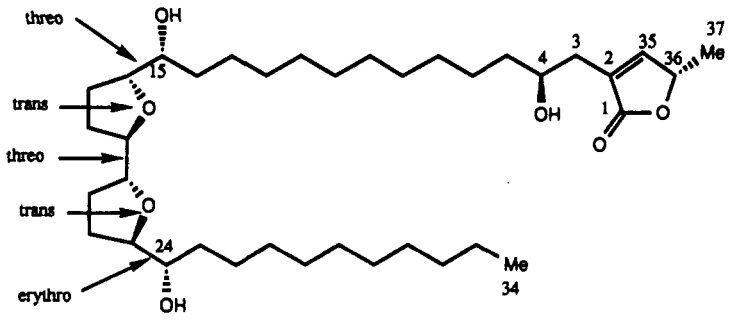
In addition to the resonances due to the oxygenated carbons of the lactone and the three hydroxylated carbons at δ 74.86, 74.71, and 70.19, the ^{13}C-nmr spectrum showed two resonances at 83.56 and 83.49, also due to oxygen-bearing carbons. These ^{13}C-nmr resonances and their corresponding ^{1}H-nmr resonances at δ 3.79 were directly analogous



6



7



13

to similar signals in annonacin (3), goniothalamicin (4), and annoreticuin (1), indicating the presence of a single tetrahydrofuran moiety. The placement of the two OH groups alpha to the tetrahydrofuran functionality was established by ^{1}H-^{1}H decoupling experiments that linked the two-proton signal at δ 3.40 to another two-proton signal at δ 3.79. The subsequent downfield shift of the signal at δ 3.40 (m) to 4.86 (m) in the ^{1}H-nmr spectrum of the acetate derivative confirmed this assignment. The presence of an additional carbonyl at 1695 cm^{-1} in the ir spectrum of **3** suggested that **3** was a keto analogue of annoreticuin [**1**]. This phenomenon was also evident by the presence of a carbonyl resonance at δ 213.46, in addition to C-1 at δ 176.31 in the ^{13}C-nmr spectrum. The positions of the tetrahydrofuran ring and the keto group along the hydrocarbon chain were determined by careful analysis of the eims, cims, and fabms fragments of **3** and its acetyl derivative and TMSi derivative (Figure 1).

The eims of the TMSi derivative of **3** (mol wt 810) produced intense ions at *m/z* 271, 341, 469, and 539 and corresponding signals in the eims of **3**, which clearly positioned the tetrahydrofuran ring at C-16 along the hydrocarbon chain and allowed the assign-

TABLE 1. Nmr Spectral Data (^{1}H nmr 200 MHz, ^{13}C nmr 50.3 MHz, $CDCl_3$) of Compounds **3** (δ of ^{1}H and ^{13}C nmr) and Triacetyl **3** (δ of ^{1}H nmr), δ Scale, Relative to TMS.[a]

Position	**3**		Triacetyl **3**
	^{1}H	^{13}C	^{1}H
1	—	176.31 s	—
2	—	132.22 s	—
3	3a 2.45 m 3b 2.51 m	37.42 t	3a 2.52 m 3b 2.57 m
4	3.88 m	70.19 d	5.10 m
5–7	1.26–1.61 m	22–38 t	1.26–1.61 m
8	2.42 t (6.2)	43.14 t	2.39 t (6.2)
9	—	213.46 s	—
10	2.40 t (6.2)	43.02 t	2.38 t (6.2)
11–14	1.26–1.61 m	22–38 t	1.26–1.61 m
15, 20	3.40 m	74.86, 74.71 d	4.86 m
16, 19	3.79 m	83.56, 83.49 d	3.97 m
17, 18	1.69, 1.97 m	22–38 t	1.69, 1.97 m
21–31	1.26–1.61 m	22–38 t	1.26–1.61 m
32	0.88 t (6.8)	14.34 q	0.88 t (6.8)
33	7.21 d (1.4)	153.45 d	7.09 d (1.4)
34	5.08 qd (6.8, 1.4)	78.82 d	5.02 qd 6.8, 1.4)
35	1.44 d (6.8)	19.34 q	1.44 d (6.8)
4–OAc			2.03 s
15,20–OAc			2.08 s

[a] *J* (in Hz) in parentheses.

ment of the OH groups at C-15 and C-20 relative to the tetrahydrofuran ring. The position of the remaining isolated carbonyl group at C-9 was illustrated by fragments in the eims of **3** and its acetyl and TMSi derivatives at *m/z* 225, 267, and 297,

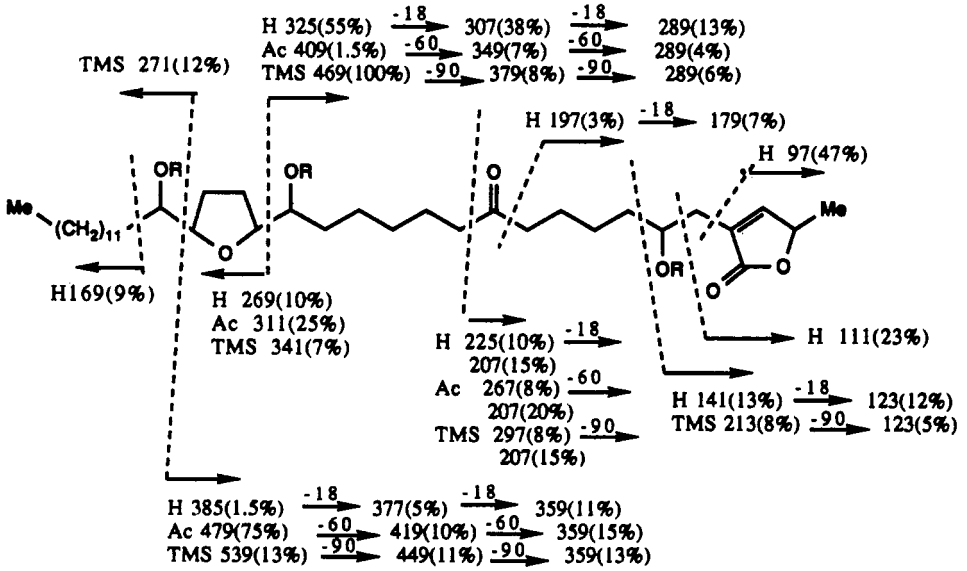


FIGURE 1. Diagnostic eims fragment ions (*m/z*) of annoreticuin-9-one [**3**]. "R" designates the underivatized material (H), the acetyl derivative (Ac) and the trimethylsilyl derivative (TMSi); the ions of *m/z* −18, *m/z* −60 and *m/z* −90 are indicative of the losses of H_2O, HOAc, and TMSiOH, respectively.

respectively. These fragments showed a loss of H_2O, HOAc, and TMSiOH respectively, to give *m/z* 207. In addition to the diagnostic fragment ions shown in Figure 1, the eims spectrum of **3** showed a peak at *m/z* 240 (2.4%), which could easily be explained by the formation of the ion $[CH_2=C(OH)]-C_{11}H_{17}O_3]^+$ as a McLafferty rearrangement from the $[M]^+$ ion. Formation of such an ion is conclusive proof for the position of the keto group at C-9 (5).

The ^{1}H-nmr data also supported the presence of the ketone at C-9, since the signal at δ 3.60 in **1**, which was assigned to the proton on the carbon attached to the 9-OH group of annoreticuin, was missing in **3**. Two additional two-proton triplets (J=6.2 Hz) were seen in the spectrum of **3** at δ 2.40 and 2.42, consistent with two methylene groups flanking the keto group at C-8 and C-10.

The relative stereochemistry of the tetrahydrofuran ring and the two adjacent hydroxyls at C-15 and C-20 was deduced by close examination of the nmr data. In the spectrum of annoreticuin-9-one [**3**], the ^{13}C-nmr chemical shifts of C-15 and C-20 at δ 74.71 and 74.86 clearly showed a threo relationship between C-15/C-16 and a threo relationship between C-19/C-20 (1,2). These assignments are based on the ^{13}C-nmr chemical shifts of a pair of model monotetrahydrofuran compounds with adjacent OH groups of the threo and erythro configuration (2). In annoreticuin-9-one triacetate, the ^{1}H-nmr chemical shifts of H-16 and H-19 at δ 3.97 indicated the trans relationship for H-16/H-19(2). Thus, the relative configuration of threo, trans, threo from C-15 to C-20 is evident. From the above data, we concluded that the structure of annoreticuin-9-one is as illustrated for **3** with the absolute and certain relative stereochemistries remaining undefined. The absolute configurations of several acetogenins have recently been deduced (6). To our knowledge, squamone [**4**] (5) is the first example of an acetogenin containing the 9-keto-monotetrahydrofuran skeleton, and annoreticuin-9-one [**3**] represents the second (2,7); indeed, **4** appears to be the ketolactone derivative of **3**.

The known acetogenin compounds, squamone [**4**] (5), solamin [**5**] (8), annomonicin [**6**] (9), and rolliniastatin 2 [**7**] (10), were readily identified by comparison of their spectral data (uv, ir, ms, ^{1}H and ^{13}C nmr) with those in the literature. Moreover, **3** was converted to **4** by treating sequentially with base to hydrolyze the lactone and with acid to recyclize (11). The reaction products showed two major components (tlc), and one was identical to **4**. Resolution on a Si gel microcolumn gave **4**, which was identical (mixed tlc, cims, and ^{1}H nmr) with **4** isolated from the plant material.

In the last ten years, annonaceous acetogenins have emerged as a new class of cytotoxic compounds, and they are under evaluation as potential anticancer agents. The cytotoxic activity of four members of the annoreticuin-series (compounds 1–4 oxygenated at C-9) and the other three known acetogenins, isolated for the first time from *A. reticulata*, were compated with four acetogenins from the annonacin-series (compounds 8–11 oxygenated at C-10 isolated from *Annona densicoma* (12, 13) (Table 2). The following interesting conclusions are observed. (a) These compounds have demonstrated selective cytotoxicity in human cell lines. (b) The annonacin series showed greater cytotoxicity than the annoreticuin series, but the later series showed better selectivity in the A-549 cancer cell line. (c) The different positions, even at adjacent sites, of the oxygen-bearing groups along the alkyl chain, lead to variation in the cytotoxicity. (d) The cytotoxicities of the bis THF (tetrahydrofuran) compounds are, in general, greater than the mono THF ones. (e) The length of the alkyl chain may affect the cytotoxicity. Some similar conclusions have been recently published in a review on these compounds (14).

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES—$[\alpha]^{25}$D values were measured on a Jasco model dip-181 digital

TABLE 2. The Cytotoxicity (ED_{50}, μg/ml) of Compounds **1–7** from *Annona reticulata* and Some Related Acetogenins **8–13** from Other Annonaceous Plants.

Compound	Cell line				
	P-388	A-549	KB	HT-29	MCF-7
Annoreticuin [**1**]	1.0	4×10^{-1}	3.37	2.28	—
Isoannoretocuin [**2**]	3.6	4×10^{-1}	6.96	3.06	—
Annoreticuin-9-one [**3**]	2×10^{-1}	10^{-2}	4.66	1.32	—
Squamone [**4**]	5.6	1.34	—	1.50	2.14
Solamin [**5**]	4×10^{-2}	—	3×10^{-1}	—	—
Annomonicin [**6**]	2.4×10^{-1}	—	1.73	—	—
Rolliniastatin 2 [**7**]	$<10^{-5}$	$<10^{-3}$	2.29	6.4×10^{-1}	—
Annonacin [**8**] (11)	10^{-5}	10^{-3}	10^{-4}	3	—
Isoannonacin [**9**] (11)	3	2×10^{-2}	—	2×10^{-3}	—
Annonacin-10-one [**10**] (11)	10^{-6}	10^{-1}	10^{-2}	1	—
Isoannonacin-10-one [**11**] (11)	5×10^{-1}	7×10^{-2}	—	9×10^{-3}	—
Reticulatacin [**12**] (12)	—	3.49	—	4.66	2.91
Bullatacin [**13**] (10)	—	$<10^{-5}$	—	$<10^{-3}$	$<10^{-5}$

polarimeter. The uv absorption spectra were obtained on a Beckman model 34 spectrophotometer, and ir spectra were determined on a Hitachi model 260-30 infrared spectrophotometer. The ^{1}H, ^{13}C, DEPT and 2D mnr spectra were taken on a Varian Gemini-200 spectrometer with TMS as internal standard, and chemical shifts were recorded in δ units. Ms was obtained on a Jeol JMS-D-100 mass spectrometer. Si gel 60 (Merck, 230–400 mesh) and active carbon (Wako, for cc) were used for cc; pre-coated Si gel plates (Merck, Kieselgel 60 F-254, 0.20 mm) were used for analytical tlc, and pre-coated Si gel plates (Merck, Kieselgel 60 F-254, 0.25 mm) were used for preparative tlc.

PLANT MATERIAL.—Leaves of *A. reticulata* were collected form Chie Shan, Kaohsiung, in April,1990. Voucher specimens are kept in the School of Pharmacy , Kaohsiung Medical College, Kaohsiung, Taiwan, Republic of China.

EXTRACTION AND SEPARATION.—The fresh leaves (3.0 kg) were extracted 5 times with MeOH (10 liters) at room temperature for 40 h. The combined MeOH extracts were evaporated and partitioned to yield $CHCl_3$ and aqueous extracts. The $CHCl_3$ solution was extracted with 3% HCl to remove alkaloids. The neutral $CHCl_3$ solution was dried (K_2CO_3) and evaporated to leave a brownish viscous residue (130 g). The residue was subjected to Si gel cc and eluted with *n*-hexane/$CHCl_3$/MeOH gradually increasing the polarity. The eluents were combined to 15 fractions on the basis of tlc. Using the bioassay-directed fractionation method, the active fractions 6 to 15 showed significant cytotoxicities in P-388, A-549, KB, and HT-29 tumor cell lines. Further purification by repeated chromatography over Si gel (gradients of *n*-hexane/$CHCl_3$/MeOH and *n*-hexane/EtOAc/Me_2CO), Sephadex LH-20 (gradients of *n*-hexane/CH_2Cl_2/MeOH), and active carbon (gradients of MeOH/$CHCl_3$) columns separated annoreticuin [**1**] (20 mg) and isoannoreticuin [**2**] (12 mg) (1). From the mother liquors of annoreticuin [**1**] and isoannoreticuin [**2**], annoreticuin-9-one [**3**] [35 mg, $CHCl_3$-Me_2CO-MeOH (7:1.5:0.5), R_f 0.4], squamone [**4**] [20 mg, *n*-hexane–EtOAc (7:1), R_f 0.5], solamin [**5**] [18 mg, *n*-hexane–THF (7:3), R_f 0.4], annomonicin [**6**] [8 mg, $CHCl_3$-MeOH (8:1), R_f 0.4], and rolliniastatin 2 [**7**] [14 mg, *n*-hexane–Me_2CO–MeOH (7:3:0.5), R_f 0.5] were isolated by preparative tlc on Si gel (0.25 mm, 20×20 cm^2).

Annoreticuin-9-one [**3**].—White amorphous powder (35 mg): $C_{35}H_{62}O_7$; $[\alpha]^{25}D$ +11.7° (c=0.02, $CHCl_3$); uv (MeOH) λ max 215 nm ($\epsilon=3.4\times10^3$); ir ν max cm^{-1} 3420, 2915, 2850, 1750, 1700, 1470, 1320, 1080, 1030. 960; eims (70 eV) see Figure 1; hrfabms ($[MH]^+$ 595.4535 (595.4557 calcd for $C_{35}H_{63}O_7$); ^{1}H nmr (200 MHz, $CDCl_3$) and ^{13}C nmr (50.3 MHz, $CDCl_3$) see Table 1.

Tricetylannoreticuin-9-one.—Treatment of **3** (5 mg) with Ac_2O pyridine (room temperature, overnight) and subsequent workup gave triacetyl **3** (quantitative yield) as an oil: ir ν max 1740, 1730, and 1700; eims see Figure 1; cims (isobutane) *m/z* $[MH]^+$ 721, 661, 601, 541, 479, 419, 359, 311, 207; ^{1}H nmr (200 MHz, $CDCl_3$) see Table 1.

Tris-trimethylsilylannoreticuin-9-one.—Dry micro-amount samples of **3** were treated with 20 μl of bis-(trimethylsilyl)-acetamide–pyridine (10:1) and heated at 70° for 30 min for eims and fabms determinations (Figure 1).

Conversion of annoreticuin-9-one [**3**] *to squamone* [**4**].—Compound **3** (15 mg) was treated with 2% KOH in *t*-BuOH (1 ml) [2% solid KOH pellet by weight/volume in *t*-BuOH at room temperature for 24 h; the solution was acidified with 10% HCl to pH 1–2, set aside for 30 min, and partioned between $CHCl_3$ and H_2O. Two major components were evident in the $CHCl_3$ residue (tlc); these were resolved by microchromatography over Si gel in a dispopipette ($CHCl_3$/EtOAc/ MeOH gradient). One of the compounds was identical to **4** (co-tlc, eims and ^1H nmr) (11).

Squamone [**4**].—White amorphous powder (20 mg), mp 87–89°, $[\alpha]^{25}D$ +7.0° (c=0.12, $CHCl_3$), characterized by spectral (tlc, uv, ir, eims, cims, fabms, and ^1H, ^{13}C and 2D nmr) analyses and comparison with literature data (7).

Solamin [**5**].—Small needle-like crystals (18 mg), mp 65–68°, $[\alpha]^{25}D$ +15.6° (c=0.15, $CHCl_3$), characterizd by spectral (tlc, uv, ir, eims, cims, fabms, and ^1H and ^{13}C nmr) analyses and comparison with literature data (8).

Annomonicin [**6**].—White amorphous powder (8 mg), mp 49–51°, $[\alpha]^{25}D$ +5.0° (c=0.17, $CHCl_3$), characterized by spectral (tlc, uv, ir eims, cims, fabms, and ^1H and ^{13}C nmr) analyses and comparison with literature data (9).

Rolliniastatin 2 [**7**].—Waxy solid (14 mg): mp 72–74°; $[\alpha]^{25}D$ +8.0° (c=0.2, $CHCl_3$), characterized by spectral (tlc, uv, ir, eims, cims, fabms, and ^1H and ^{13}C nmr) analyses and comparison with literature data (10).

Cytotoxicity testing.—The cytotoxicity assays were carried out according to procedures described in the literature (15, 16).

ACKNOWLEDGMENTS

This investigation was supported by a grant from the National Science Council of the Republic of China (NSC 81-0208-M-037-505) awarded to Y. C. Wu.

LITERATURE CITED

1. Y.C. Wu, F.R. Chang, C.Y. Duh, and S.K. Wang, *Heterocycles*, **34**, 667 (1992).
2. J.K. Rupprecht, Y.H. Hui, and J.K. McLaughlin, *J. Nat. Prod.*, **53**, 237 (1990).
3. T.G. McCloud, D.L. Smith, C.J. Chang, and J.M. Cassady, *Experientia*, **43**, 947 (1987).
4. A. Alkofahi, J.K. Rupprecht, D.L. Smith, C.J. Chang, and J.L. McLaughlin, *Experientia*, **44**, 83 (1988).
5. A. Hisham, L.A.C. Pieters, M. Claeys, E. Esmans, R. Dommisse, and A.J. Vlietinck, *Phytochemistry*, **30**, 545 (1991).
6. M.J. Rieser, Y.H. Hui, J.K. Rupprecht, J.F. Kozlowski, K.V. Wood, J.L. McLaughlin, P.R. Hanson, Z. Zhuang, and T.R. Hoye, *J. Am. Chem. Soc.*, **114**, 10203 (1992).
7. X.H. Li, Y.H. Hui, J.K. Rupprecht, Y.M. Liu, K.V. Wood, D.L. Smith, C.J. Chang, and J.L. McLaughlin, *J. Nat. Prod.*, **53**, 81 (1990).
8. S.H. Myint, D. Cortes, A. Laurens, R. Hocquemiller, M. Leboeuf, A. Cave, J. Cotte, and A.M. Quero, *Phytochemistry*, **30**, 3335 (1991).
9. A. Jossang, B. Dubaele, and A. Cave, *Tetrahedron Lett.*, **31**, 1861 (1990).
10. G.R. Pettit, R. Riesen, J.E. Leet, J. Polonsky, C.R. Smith, J.M. Schmidt, C. Dufresne, D. Schaufelberger, and C. Moretti, *Heterocycles*, **28**, 213 (1989).
11. Y.H. Hui, J.K. Rupprecht, Y.M. Liu, J. E. Andeson, D.L. Smith, C.J. Chang, and J.L. McLaughlin, *J. Nat. Prod.*, **52**, 463 (1989).
12. L. Xu, C.J. Chang, J.G. Yu, and J.M. Cassady, *J. Org.Chem.*, **54**, 5418 (1989).
13. J.M. Saad, Y.H. Hui, J. K. Rupprecht, J.E. Anderson, J.K. Kozlowski, G.Z. Zhao, K.V. Wood, and J.L. McLaughlin, *Tetrahedron*, **47**, 2751 (1991).
14. X.P. Fang, M.J. Rieser, Z.M. Gu, G.X. Zhao, and J.L. McLaughlin, *Phytochemical Analysis*, **4**, 27 and 49 (1993).
15. M.C. Alley, D.A. Scudiero, A. Monks, M.L. Hursey, M.J. Czerwinski, D.L. Fine, B.J. Abbott, J.G. Mayo, R.H. Shoemaker, and M.R. Boyd, *Cancer Res.*, **48**, 589 (1988).
16. S.K. Wang, C.Y. Duh, Y.C. Wu, Y. Wang, M.C. Cheng, K. Soong, and L.S. Fang, *J. Nat. Prod.*, **55**, 1430 (1992).

Received 11 January 1993