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Reactions of Withaferin-A with Model Biological Nucleophiles

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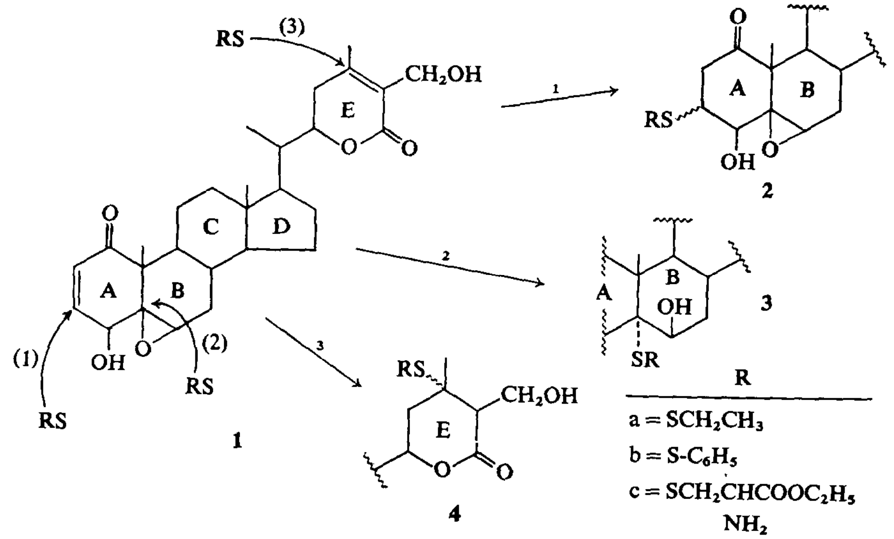
Withaferin-A was reacted with three model biological nucleophiles: ethyl mercaptan, thiophenol, and L-cysteine ethyl ester. Under neutral or alkaline conditions, each of the thiols underwent facile Michael addition with the antitumor agent. The most chemically reactive site for Michael Addition reactions with the model thiols was the unsaturated A-ring of withaferin-A. The possible significance of this reaction with regard to antitumor activity is discussed.

INTRODUCTION

Withaferin-A (**1**) is a polyfunctional steroid-lactone isolated from the leaves of *Withania somnifera*, Dun. (*1–3*), and from *Acnistus arborescens* (*4*, *5*). This compound has demonstrated antitumor activity in a number of animal test systems including the sarcoma 180 (SA-180) and Ehrlich ascites carcinoma in mice (*5*, *6*). Withaferin-A is a mitotic inhibitor causing metaphase arrest in plant and animal cells (*6–8*), and it thus behaves like the *Vinca* alkaloids, colchicine, and podophyllotoxin in retarding tumor growth. The steroid also inhibits protein synthesis since it reduces the uptake of [^{14}C]-phenylalanine by SA-180 cells (*9*). Withaferin-A has been grouped with other α,β-unsaturated steroid derivatives which possess the potential to bind to sulfhydryl groups of enzymes involved in critical cellular processes like amino acid transport (*10*).

The chemical mechanism by which withaferin-A accomplishes the inhibition of tumor growth is unknown. Several explanations have been proposed to account for its role in this process. In all cases, it has been presupposed that the steroid somehow binds to a critical macromolecule or enzyme active site. Mechanisms suggested include acylation (*11*), or possibly alkylation (*10*) through nucleophilic Michael addition at the E-ring unsaturated lactone position of withaferin-A. It was noted that other functionalities of withaferin-A could allow the steroid to act as a bifunctional alkylating agent (*11*). No experimental evidence has been provided to support any of the proposed mechanisms.

Withaferin-A possesses three likely positions which might be involved in *in vivo* alkylation reactions with biological nucleophiles. These include the A-ring at position 3, the epoxide at position 5 (or 6), and the E-ring at position 24. Model biological nucleophiles including cysteine and other thiols (*10*, *12*, *13*) have been shown to attack unsaturated systems like the A-ring of withaferin-A (*5*, *14*, *15*), lactones (*10*, *12*), acrylic esters (*16*), and epoxides (*17*). This report describes the results obtained when withaferin-A was reacted with three model biological nucleophiles: ethanethiol, thiophenol, and L-cysteine ethyl ester.



EXPERIMENTAL

Melting points were determined on a Thomas Hoover apparatus in open-ended capillaries and are corrected. Infrared spectra were obtained (KBr disks) on a Perkin-Elmer 267 spectrophotometer. Nuclear magnetic resonance spectra were measured in $CDCl_3$ or DMSO-D_6 (TMS internal standard) on a Varian T-60 spectrometer. Mass spectra were obtained on a Finnigan 3200 spectrometer at 70 eV using a solid sample probe inlet system; and chemical ionization spectra were obtained through the University of Virginia, Department of Chemistry. High resolution mass spectra were obtained through Battelle Columbus Laboratories, Columbus, Ohio. Elemental analyses were performed by Midwest Microlab Ltd., Indianapolis, Indiana.

Chromatography. Column chromatography separations were performed with Baker AR silica gel (No. 3405), while analytical and preparative thin-layer chromatography (tlc) separations were conducted with Merck silica gel G F-254. Thin-layer plates were prepared using a Quickfit spreader, and, after drying at room temperature for 30 min, plates were activated at 120°C for 30 min prior to use. Visualization of developed chromatograms was achieved by use of 254-nm ultraviolet light, and by spraying tlc plates with *p*-anisaldehyde:60% $HClO_4$:Me_2CO:H_2O, (0.5:5:10:40) and warming them with a heat gun.

Isolation of withaferin-A (1) from Withania somnifera, Dun. Leaves and stems (50 lb) of *W. somnifera*, Dun., were purchased from S. B. Penick, and a voucher specimen has been retained. Withaferin-A was isolated from the plant material by a modification of the procedure described by Yarden and Lavie (*1*). From 20 lb of plant material, nearly 50 g of the steroid-lactone were obtained. The isolated withaferin-A was compared to authentic withaferin-A obtained from Professor Lavie and was found to be spectrally (nmr, ir, mass spectrum) identical: mp 242–243.5°C (reported (*18*) 243–245°C); ir (KBr disk) 2.93, 3.40, 5.92, 7.19 μm. *Anal.* Calcd for $C_{28}H_{38}O_6$: C, 71.46; H, 8.14. Found: C, 71.48; H, 7.94. Diacetate mp 199–200°C (reported (*17*) 201°C). Anal. Diacetate, calcd for $C_{32}H_{42}O_8$: C, 69.29; H, 7.63. Found: C, 69.50; H, 7.80. The chromatographic behaviors of authentic and isolated withaferin-A were identical.

Reaction of withaferin-A with ethylmercaptan: 2-hydro-3-thioethylwithaferin-A (***2a***) A solution of 1 ml of ethyl mercaptan in 10 ml of deionized H_2O was adjusted to pH 10.9 with 10% aqueous KOH. A portion (3 ml) of the mercaptan solution was added to 300 mg (0.64 mmole) of (**1**) in 25 ml of THF. Immediately after the addition of mercaptan solution was complete, the reaction mixture was diluted with 50 ml of H_2O, and extracted with EtOAc. The EtOAc extract was dried over anhydrous $MgSO_4$ and evaporated to dryness under reduced pressure. A clear gum resulted which solidified to a white foam under vacuum (0.5 mm Hg). A trace of ethyl mercaptan could be detected in the product by tlc. Prolonged heating of the foam under vacuum in a drying pistol and crystallization from EtOAc gave the adduct (**2a**), 245 mg, 74% yield; mp 243–246°C (with softening occurring at 120°C); ir (KBr) 2.82, 2.99, 5.85, 5.98 μm; uv, λ_{max} (EtOH) 220 nm ($\varepsilon = 8786$); nmr ($CDCl_3$) δ 1.27 (*t*, 3H, $J = 6.5$ Hz, Me of C_2H_5S–), 2.52 (*q*, 2H, $J = 6.5$ Hz, methylene of C_2H_5S–); *Anal.* calcd for $C_{30}H_{44}SO_6$: C, 67.61; H, 8.35; S, 6.02. Found: 67.60; H, 8.29; S, 6.25. Mass spectrum, *m/e* (relative abundance) 532 (4%), 471 (7%, M–C_2H_5S), 453 (5%), 392 (9%), 386 (11%), 336 (22%), 219 (41%), 205 (100%), 197 (90%).

Ethyl mercaptan reacted slowly with (**1**) in the absence of base, even when a 10-fold excess of the thiol was added. After 24 hr under such conditions, approximately equal amounts of (**2a**) and (**1**) (tlc analysis) were present in the reaction. The addition of a few drops of 10% aqueous KOH to the reaction mixture rapidly brought it to completion.

Reaction of withaferin-A with thiophenol: 2-Hydro-3-thiophenylwithaferin-A (***2b***). An aqueous thiophenol solution was prepared by adjusting a mixture of thiophenol (1.0 ml) and water (10 ml) to pH 12.8. This homogeneous mixture was added in 0.25-ml aliquots to a stirred solution of 300 mg of (**1**) (0.64 mmole) in 25 ml of THF until tlc analysis indicated that the reaction was complete. The clear solution was diluted with 50 ml of H_2O and was extracted twice with 50 ml of CH_2Cl_2. After separation, the organic phase was dried over anhydrous $MgSO_4$ and concentrated under vacuum to give a gummy foam which solidified upon cooling. The impure foam was purified by column chromatography over 30 g of silica gel using EtOAc:C_6H_6 (85:15) as eluent. Fractions of 7–8 ml were collected at a flow rate of about 2 ml/min. After a 50-ml forerun, fractions 4–8 consisted mostly of thiophenol; fractions 9 and 10 contained traces of (**2b**), while fractions 11–18 were pure thiophenol adduct (**2b**). The combined fractions were evaporated to yield 226 mg (63%) of (**2b**) as a white powder: mp 128–134°C (with softening at 110°C); ir (KBr disk) 2.9, 3.40, 5.92, 6.32, 7.15 μm; uv, λ_{max} (EtOH) 214 nm (11,682), 260 nm (6528); nmr ($CDCl_3$) δ 7.35 (*m*, 5H, C_6H_6S); EI mass spectrum, *m/e* 580 (molecular ion), 471 (M^+–109, or C_6H_5S); high resolution mass spectrum, *m/e* 471.2733 (calcd for $C_{28}H_{39}O_6$, 471.2746), *m/e* 109.0108 (calcd for C_6H_5S, 109.0112).

It was found in later reactions that (**2b**) could be obtained by adding the thiophenol directly to a solution of (**1**) in THF. Analogous workup including column chromatography gave (**2b**) in 57% yield. It was necessary to perform reaction workup as quickly and as simply as possible due to the instability of the thiophenol adduct.

Reaction of withaferin-A with L-*cysteine ethyl ester: 2-Hydro-3-(2-amino-2-ethylcarboxyethylthio)-withaferin-A* (*2c*). An aqueous solution of L-cysteine ethyl ester was prepared by adjusting a solution of 1.0 g of the ester in 10 ml of H_2O to pH 7.0, with 10% aqueous KOH. A portion (2 ml) of this solution was added to 200 mg of (**1**) (0.43

mmole) in 20 ml of THF. After stirring the reaction mixture for 2 min, it was diluted with 50 ml of H_2O and extracted with EtOAc. The EtOAc layer was washed once with 10% HCl, and twice with H_2O and dried over anhydrous Na_2SO_4 before being evaporated to dryness under vacuum. The resulting pale yellow gum would not crystallize upon chilling. Heating under vacuum in a drying pistol gave a glassy foam, mp 85–95°C (with softening and yellowing); ir (KBr disk) 2.86, 2.99, 3.38, 5.88, 6.2, 7.14 μm. Ultraviolet data were not determined due to the instability of the compound in solution. Analytical tlc using EtOAc gave R_f values of 0.1 for (**2c**) and 0.6 for (**1**). The chemical ionization mass spectrum (Argon/water) gave a molecular ion at 638 ($M + H_3O^+$); it was impossible to obtain EI mass spectral data on (**2c**). Likewise, elemental composition data were unobtainable on the unstable cysteine adduct. Molecular weight determination by osmometric methods (Midwest Analytical Labs, Ltd., Indianapolis, Indiana) gave an average value of 595 on two separate analyses (expected M W of (**2c**), 619). Nuclear magnetic resonance signals characteristic of the unsaturated A-ring of (**1**) were absent in the spectrum of (**2c**).

RESULTS AND DISCUSSION

For all reactions (**1**) was dissolved in THF due to its extreme insolubility in aqueous media. In initial studies, no reaction could be observed when L-cysteine was mixed with (**1**) either in acidic or basic medium. L-Cysteine ethyl ester, on the other hand, reacted rapidly and completely with (**1**) under neutral or slightly alkaline conditions. The use of the ethyl ester of cysteine apparently avoided the formation of unknown decomposition products which could be observed chromatographically in reactions conducted with L-cysteine itself. The product obtained with L-cysteine ethyl ester (**2c**) was chromatographically pure but would not crystallize. This product was extremely sensitive to silica gel and would decompose when an EtOAc solution of (**2c**) was stirred with a suspension of silica gel. Solutions of the product gradually yellowed on standing, and the solid product also darkened gradually. Rapid reaction workup was essential in the isolation of the Michael addition product (**2c**).

The Michael adduct obtained with thiophenol would decompose slowly in solution, particularly in the presence of silica gel. The ethyl mercaptan adduct (**2a**) was the most stable of the Michael addition products obtained. It was possible to obtain elemental analyses and to store the compound without chromatographic evidence of decomposition.

Assignment of the site of thioalkylation of withaferin-A (**1**) was based on the disappearance of characteristic signals in the nmr spectrum of (**1**). A portion of the nmr spectrum of (**1**) is shown in Fig. 1. The signals assigned to the vinyl protons at positions 2 and 3 of the A-ring are as follows: C-2 protons, doublet at 6.19 δ, $J = 10$ Hz; C-3 proton, 6.95 δ, doublet of doublets, $J = 10$ Hz, $J = 6$ Hz (*2*). These characteristic nmr signals were absent in the nmr spectrum of each of the adducts. With the thiophenol adduct (**2b**), it was possible to observe the aromatic protons which appeared as an unresolved multiplet at 7.35 δ. The ethyl mercaptan adduct exhibits a triplet at 1.27 δ (partially obscured by the signal for protons on C-19) and a quartet at 2.62 δ. Both of these signals may be assigned to the attached ethyl group of (**2a**). The assignment of the structure of (**2c**) is based solely on the absence of the 2 and 3-H signals characteristic of the A-ring of (**1**).

Low resolution mass spectral data gave the expected molecular weights for the ethyl mercaptan adduct (**2a**), 532, and for the thiophenol adduct (**2b**), 580, but it was impossible to obtain electron impact spectra for the L-cysteine ethyl ester adduct. Chemical ionization mass spectral data yielded an ion of mass 638 for (**2c**) indicative of the addition of H_3O^+ to the expected molecular weight of 619. Molecular weight data on (**2c**) based on colligative properties indicated a value of 595 for the adduct. The adduct also gave a positive reaction with ninhydrin reagent indicating that the amino group of L-cysteine ethyl ester was free.

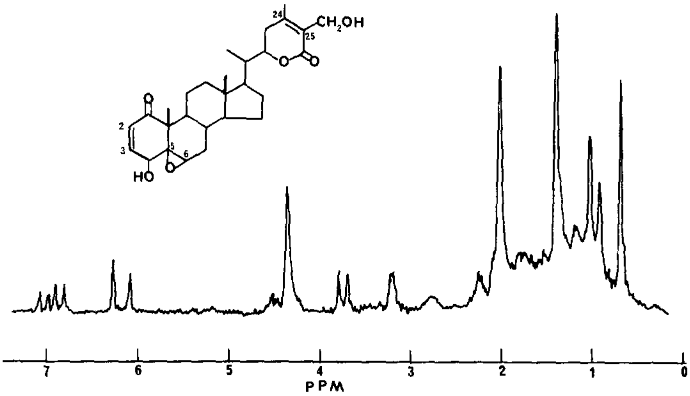


FIG. 1. Nuclear magnetic resonance spectrum of withaferin-A (1) in $CDCl_3$ (TMS internal standard).

Retro-Michael addition reactions occur when L-cysteine reacts with α, β-unsaturated lactones (*12*). Withaferin-A thio addition products exhibited instability, and the cysteine ethyl ester adduct (**2c**) was the least stable product of all. It is conceivable that withaferin-A and other antitumor agents may serve as reversible alkylating agents, particularly if they undergo thioalkylation reactions *in vivo*.

Neither the epoxide nor the E-ring lactone was involved in reactions with ethyl mercaptan, thiophenol, or L-cysteine ethyl ester. Increasing the substitution at α, β-unsaturated lactones has been shown to diminish cytotoxicity in the case of γ-lactones (*19*) and to reduce their reactivity in Michael addition reactions (*12*). It has also been shown that endocyclic double bonds were less likely to be involved in Michael addition reactions than exocyclic systems (*20*). The fact that the E-ring of (**1**) contains a tetra-substituted, endocyclic double bond probably accounts for its lack of activity with sulfhydryl reagents in our experiments.

It is possible that *in vivo* conditions prevail wherein either the epoxide or the lactone ring may be involved in alkylation reactions, although this study indicated that the most chemically reactive site for Michael addition thioalkylation reactions is the A-ring of withaferin-A. The chemical reactivity of the A-ring of (**1**) is further evidenced by its ready reduction with hydrogen over palladium on carbon (*18*) and by the ease with which it undergoes Michael addition with methanolic sodium acetate to give 2,3-dihydro-3-methoxywithaferin-A (*5*). Numerous compounds with structural features similar to the A-ring of withaferin-A possess antitumor or cytotoxic activity (*22*). Although the role of the A-ring double bond (and possibly the epoxide) of withaferin-A in antitumor and/or cytotoxic activity is unclear, it is interesting that an A-ring analog of withaferin-A, 5β,6β-epoxy-1-oxo-cholest-2-en-4β-ol exhibits sarcoma 180 activity in mice (*21*). Further studies are being conducted to elaborate on the need for the epoxide and lactone rings for antitumor activity of withaferin-A.

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