PRELIMINARY STUDIES OF THE BIOSYNTHESIS OF AUSTIN

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Based on an M.S. thesis submitted to Iowa State University

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Preliminary studies of the biosynthesis of austin

by

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INTRODUCTION

Members of the genus Aspergillus produce an amazing variety of secondary metabolites. Some of these metabolites have become a cause of great concern due to their high toxicity or carcinogenicity. A number of cases of livestock toxicoses due to fungal metabolite contaminated feeds have been documented (1), and there is evidence that the high incidence of liver cancer in some parts of the world may be due to the ingestion of aflatoxin B₁, the most potent hepatocarcinogen known (2). Not all Aspergillus metabolites are detrimental, however, some have been found to be useful antibiotics.

Aspergillus ustus is one of the most prevalent fungi in the soil. There are now two reports in the literature of the occurrence of toxin-producing strains of this fungus on stored foodstuffs. In addition, strains of A. ustus have been isolated along with Penicillium species from samples of South African cheeses (3). All A. ustus isolates tested were judged to be highly toxic to ducklings when grown on maize meal, however, the toxins involved were not isolated. Austin is the trivial name of one of the toxins made by the fungus found on stored food. Preliminary work to studying the biosynthesis of this compound using ¹³C-labeled sodium acetate is reported here. The feasibility of the biosynthetic

study was determined by feeding $[1-^{14}\text{C}]$ -sodium acetate to

A. ustus cultures.

HISTORICAL

The Fungus and its Secondary Metabolites

The fungus now referred to as Aspergillus ustus was first described by M. Bainier in 1881 (4). A more complete description of the fungus, including various strains, was given in 1926 by Thom and Church (5). Five species are now recognized by Raper and Fennell as belonging to the A. ustus group (6). By far the most prevalent species is A. ustus (Bainier) Thom and Church. It is known to be very common in the soil and on decaying vegetation. The other four species, which are mainly tropical, are A. puniceus Kwon and Fennell, n.sp.; A. Panamensis Raper and Thom; A. conjunctus Kwon and Fennell, n.sp.; and A. deflectus Fennell and Raper.

Reports of antibacterial activity in extracts of A. ustus (Bainier) Thom and Church appeared in the 1940's (7, 8, 9). However, the fungus used in these studies was later reclassified as a member of the species A. nidulans (10).

In 1951, Raistrick and Stickings isolated an acid, melting point 169-170°C, from four strains of A. ustus (11). They determined its structure (1) by chemical methods and proposed the name ustic acid for the compound. No type of biological activity was given for it. Since then, it has also been found in Paecilomyces victorinae and Ustilago zeae (12). The well-known fungal toxin kojic acid (2) has also been reported in A. ustus (13).

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The screening of fungi for toxigenic metabolites in recent times has resulted in the discovery of many new products of A. ustus. In the early 1970's, P.S. Steyn determined that a strain of A. ustus (Bainier) Thom and Church (C.S.I.R. 1128), which was isolated from stored foodstuffs, produced toxic principles when grown on maize meal. One of the toxic metabolites was called austamide (14). Its spectral and chemical data were consistent with structural formula 3. A related metabolite, prolyl-2-(1',1'-dimethylallyl)tryptophyldiketopiperazine (4) (15) was also isolated. It is a possible biogenetic precursor of austamide. Four other diketopiperazines have been extracted from the fungus and identified (16, 17). Vleggaar, Steyn, and Nagel reported that the major toxin from the same strain of A. ustus was austdiol (5) (18). It was found to be a gastrointestinal toxin, but no lethal dosage was given. The structure was elucidated by chemical and spectral methods. The conformation and stereochemistry were later confirmed by x-ray crystallographic analysis of the 5-bromo derivative (19). Dihydrodeoxy-8-epi-austdiol is also produced

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by the fungus (20). Six minor metabolites of the same fungus were found to be interrelated (21). The general name of austocystin was proposed for them. Austocystin A (6) is shown below. Austocystins B, C, D, and E do not have a chlorine in the 7-position, but have a 3-hydroxyisopentanyl group in the 9-position.

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The biogenesis of the austocystins is probably related to the biogenesis of the aflatoxins. Averufin (7) and small amounts of sterigmatocystin (8) have been isolated from A. ustus cultures (22). Both have been shown to incorporate efficiently into aflatoxin B₁ (9) (23, 24, 25). Versicolorin C (10) is also found in A. ustus (21) and appears to be related to the aflatoxins (26).

In 1976 Chexal et al. (27) reported that a strain of A. ustus (NRRL 6017) isolated from stored black-eyed peas produced a toxic metabolite for which the name austin was proposed. To obtain the compound, the fungus was grown in Fernbach flasks on a medium made of 100 g shredded wheat, 200 ml Difco mycological broth, 2% (by weight) yeast extract,

and 15% sucrose. The isolation of austin, the major toxin, involved chloroform extraction of the contents of the flask, and extensive chromatography of the extract on silica gel and neutral alumina. Death occurred in day-old cockerels when they were administered 375 mg/kg amounts orally. Thorough testing of austin was not done due to lack of material. Spectral data were acquired on the compound, but no substantial structural features of the molecule could be ascertained. Since the amount of available material was small, a single crystal x-ray diffraction experiment was performed. Suitable crystals were obtained by recrystallization in chloroform-methanol solutions. They belonged to the orthorhombic space group $P2_12_12_1$ with $a = 7.774$ (1), $b = 15.092$ (2), and $c = 21.243$ (2) Å. The structure (11) was solved by direct methods, and the final discrepancy index was 0.033 for the 1,864 diffractometer observed reflections.

The largest ion observed in the mass spectrum of austin (16 ev electron impact) was 442.163, which represents a loss of C_3H_6O from the parent ion. The $\lambda_{\text{max}}^{\text{EtOH}}$ of the uv spectrum

was 243 nm (ϵ , 11,900). Other spectral data including pmr, ^{13}C -nmr, and ir, are given in Tables 1, 2, and 3 respectively. No individual assignments were made in the ^{13}C -nmr. It was noted at the time that austin bore no resemblance to any metabolites previously obtained from A. ustus. Rather it was similar to andibenin (12), a metabolite of Aspergillus varicolor (28). Both have the spirofused α,β -unsaturated δ -lactone, and possibly are sesterterpenoids (27).

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The Sesterterpenoids

The sesterterpenoids represent a rare group among the approximately 4,000 known terpenoids (29). They are formed from five isoprene units, and are biosynthetically derived from geranylfarnesyl pyrophosphate (13).

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Table 1. Proton nmr spectrum of austin (11).

Chemical ^a Shift (ppm)	Multiplicity ^b Coupling constant (Hz)	Tentative Assignment
1.24	s	C(23)H ₃
1.42	s	C(28)H ₃
1.58	s	C(35)H ₃
1.66	s	C(36)H ₃
1.92	s	C(34)H ₃
2.06	s	C(33)H ₃
1.34	d J=6.8	C(25)H ₃
4.46	q J=6.8	-C(11)H
3.64	es	-O(26)H
5.52	d J=1.5	=C(29)H ₂
5.74	d J=1.5	
6.05	s	-C(17)HOAc
6.65	d J=10	=C(3)H
6.10	d J=10	=C(2)H-C(=O)-
1.5-1.9	m	-C(6)H (pro-s)
		-C(5)H ₂
3.24	m	-C(6)H (pro-R)

^aDownfield from TMS.^bs=singlet, d=doublet, q=quartet, es=exchangeable singlet
m=multiplet.

Table 2. ^{13}C -nmr broadband decoupled spectrum of austin (11)^a.

Carbonyl carbons	sp^2 hybridized carbons	sp^3 hybridized carbons adjacent to oxygens	sp^3 hybridized carbons
171.0	146.6	85.6	62.9
170.1	143.8	84.2	46.7
168.5	137.6	80.7	42.2
163.8	132.7	78.8	27.1
	120.2	74.8	26.7
	118.2		26.0
			23.7
			22.5
			20.7
			20.4
			15.5
			11.5

^aAll shifts reported in ppm downfield from TMS.

Table 3. Infrared spectrum (KBr) of austin.

Major Band cm^{-1}	Tentative Assignment
3490	OH
1780	γ -lactone
1747	acetate
1740	δ -lactone
1710	α, β -unsaturated δ -lactone

At this time, only five structural types are generally accepted in this category. They include the linear, ophiobolane, cheilanthatriol, retigeranic acid, and gascardic acid skeletal types. The linear sesterterpenes have been isolated from various sources. All-trans-geranylfarnesol has been found in insect wax, while all-trans-geranylnerolidol is made by the fungus Cochliobolus heterostrophus. Quite a few linear sesterterpenes, characterized by the presence of furan rings and sometimes a tetronic acid moiety have been obtained from marine sponges.

The linear skeletal type and the ophiobolane are the only groups which have more than a few members. Ophiobolin A (14) was the first of the ophiobolane type to be isolated.

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It was found in the fungus Helminthosporium oryzae. Studies have been made of the biosynthesis of the ophiobolins. When $[2-^{14}\text{C}]$ -mevalonic acid was fed to Cochliobolus miyabeanus, ophiobolin A was obtained six days later which showed an incorporation of 2.48%. The labeling pattern expected by the following cyclization mechanism was observed:

A number of other biosynthetic studies have been done on the ophiobolins. An interesting study by Nozoe has shown that $[1,1-3H_2]$ -all-trans-geranylfarnesyl pyrophosphate incorporated to the extent of 20% into ophiobolin F (15) using a cell-free system from Cochliobolus heterostrophus.

The third type of sesterterpene skeleton is the cheilanthatriol (16) type. It has been obtained from the fern Cheilanthes farinosa. Also in this group is the sponge

metabolite scalarin (17). No biosynthetic studies have been done on these compounds.

Retigeranic acid (18), the fourth structural type, has been obtained from lichens. No other compounds of this class have been isolated.

Gascardic acid (19), the first known sesterterpene, was discovered in 1965 (30). It was found in the lac of the insect Gascardia madagascariensis. Again, no other compounds having this type of structure are known.

Austin (11) has a different skeleton from all of the known sesterterpenes and may represent a new structural type. To determine if this is indeed the case, or if on the other hand it is a degraded triterpene, a biosynthetic study would be required.

Methods of Studying Biosynthesis

There are a number of ways that information can be obtained on the biosynthesis of a given metabolite. The ideal approach is to identify intermediates in a metabolic pathway, and then use purified enzymes and isotopically labeled precursors to study the mechanism of each reaction in the pathway (31). In practice, this is often a very difficult task. The use of cell-free systems has not been generally successful in studying secondary pathways (32). The reason for this is not known, although it has been proposed that the destruction of enzyme associations, perhaps ones connected with structural elements of the cell, may be involved. When cell-free systems have been employed successfully, the results have been impressive. The previously cited work by Nözoe is one example of this. Another interesting example is Deyrup and Allen's study of a partially purified enzyme of Aspergillus amstelodami (33). This enzyme is known to transfer an isoprene unit from 3-methyl-2-butenyl-1-pyrophosphate to cyclo-L-alanyl-L-tryptophanyl. They found it equally active in transferring an isoprene unit to cyclo-L-prolyl-L-tryptophanyl. The product of the reaction appeared to be prolyl-2-(1',1'-dimethylallyl)tryptophyldiketopiperazine. Their results suggest that the biosynthesis of this compound in A. ustus may involve a similar isopreylation.

Most studies of secondary biosynthesis have been done in vivo. One type of experimental approach to this utilizes mutant organisms (34). Organisms are obtained which have one defective gene which prevents the synthesis of an enzyme. When this enzyme is involved in the biosynthetic pathway of interest, the final secondary metabolite may not be formed. Instead the intermediate preceding the defective step will accumulate, thus allowing its isolation and identification. This is how averufin (7) was first postulated as a precursor to aflatoxin B₁ (9) (23). When the product of the defective step is supplied, it may still be carried on to the end product. Possible precursors may be tested in this way. However, experiments with mutant organisms have been less successful in elucidating secondary pathways than primary pathways. There are three difficulties commonly encountered. One difficulty is the isolation of the mutant organisms. Organisms having defective primary metabolism cannot grow, and can be separated from non-mutants using antibiotics. Organisms defective in secondary pathways can only be isolated by plating them out and screening for a mutation. Another difficulty is that enzymes in secondary pathways often catalyze reactions on different substrates, and a defect in the enzyme will have multiple effects. Thirdly, the accumulated intermediate preceding a defective step may be a suitable substrate for other enzymes, and may be transformed into another compound.

Another type of biosynthetic experiment in vivo involves monitoring the various compounds produced by an organism as time progresses (35). In this way, it may be possible to observe intermediate steps in the formation of a final product, and accordingly form a hypothesis about its biogenesis. In studies on microorganisms, the metabolism may be too rapid to use this method rigorously. Nevertheless, a number of metabolites isolated at the same time may be related, either as precursors and products, or as products of a common intermediate. These may give a clue to the biogenesis of the product of interest. For example, the structural similarity between sterigmatocystin (8) and aflatoxin B₁ (9), and the presence of sterigmatocystin derivatives and aflatoxin B₁ in A. flavus, caused some people to suspect that a difuroxanthone was an intermediate in aflatoxin biosynthesis (36). The incorporation of sterigmatocystin into aflatoxin B₁ was later demonstrated using isotopic labeling.

One of the most important methods of studying the biosynthesis of a secondary metabolite is the precursor-incorporation experiment. In this type of experiment, a suspected precursor may be tested by determining the efficiency with which the isotopically labeled precursor incorporates into the product. Alternatively, the biosynthesis of a metabolite may be deduced by observing how a number of labeled precursor molecules are arranged in the final product.

In the past, radioactive isotopes such as ^{14}C and ^3H have commonly been employed in such studies. The use of radioisotopes has a number of advantages, the most important being the high sensitivity with which they can be detected (37). Thus, only tracer amounts of precursor need be fed, and the disruption of normal metabolism is kept to a minimum. In addition, the detection equipment is relatively inexpensive, and a wide variety of labeled precursors are now commercially available at reasonable prices.

The location of the ^{14}C and ^3H labels in the metabolite, however, may be difficult. It involves the exacting chemical degradation of the molecule by known schemes. Depending on the structure and complexity of the metabolite, this can be a formidable task. Error may be introduced if fragments are not isolated cleanly. In some cases, another type of labeling technique may therefore be desirable.

The stable isotopes ^{13}C and ^2H have been used as alternatives to the corresponding radioisotopes. The use of ^{13}C was previously hampered, though, by the lack of an adequate method of detection. Mass spectral analysis has sometimes been used, but many molecules have fragmentation patterns too complex to allow determination of labeled sites with certainty. In the 1960's, a technique was explored by which ^{13}C atoms were located by their satellite peaks in the proton nmr. This approach is limited in that only carbons bearing

protons could be assayed (38). It was not until significant advances in nmr spectroscopic instrumentation were made, that ^{13}C labeling became important. ^{13}C like the proton has a spin of $1/2$, and the chemical shift range of ca. 200 ppm provides good resolution of signals in most average-sized molecules. However, the natural abundance of 1.1%, the presence of ^{13}C - ^1H coupling, and a smaller magnetic moment cause the ^{13}C nucleus to be about 5700 times less sensitive in nmr spectroscopy than the proton. The pulsed Fourier transform technique and broadband proton decoupling have alleviated this problem somewhat, and made it possible to do routine ^{13}C -nmr on as little as 50 μmoles of material (39). The importance of this to the study of biosynthesis can be seen in the large number of publications which have already appeared in this area.

The major advantage of labeling with ^{13}C is that no chemical degradation of the metabolite is necessary to locate the label. Once the natural abundance ^{13}C -nmr spectrum of the compound is assigned, labeled sites can be located by observing peak enhancements in the ^{13}C -nmr spectrum of the enriched material. In addition, precursors labeled in various positions may be used with little more effort than using a single labeled precursor. Doubly labeled precursors, such as $[1,2-^{13}\text{C}_2]$ -sodium acetate, can be used to follow incorporation of intact units, since they produce observable

^{13}C - ^{13}C coupling in the ^{13}C -nmr spectrum. This is analogous to the use of multiple labeling in radioisotope experiments.

Sensitivity is still a limiting factor in ^{13}C -nmr biosynthetic studies. Because of the variability of peak heights in the ^{13}C -nmr spectrum due to unequal nuclear Overhauser effects and differing nuclear relaxation times, relatively large incorporations of highly enriched precursors are necessary to avoid ambiguity in experimental results. This necessitates feeding more than tracer amounts of precursors to the organism. Disruption of normal metabolism may occur and care must be exercised in choosing experimental conditions and interpreting results (40).

Because of the complexity of the austin molecule and lack of information on its chemistry, it was decided that the use of ^{13}C labeling methods would be the most advantageous in studying its biosynthesis. Preliminary work to the labeling of austin with ^{13}C enriched sodium acetate is reported here. An attempt was also made to isolate precursors or compounds related to austin in order to gain insight into possible steps in its formation. Results of this are also included.

RESULTS AND DISCUSSION

Some Assignments of the ^{13}C -nmr Spectrum of AustinIntroduction

The assignment of the ^{13}C -nmr spectrum may be the most difficult part of a ^{13}C -nmr biosynthetic study. Two recent misassignments in such studies in the literature attest to the fact that great care must be taken in this area.

T. J. Simpson has stated (41):

"However, it may be seen that great caution is necessary when using model compounds for the assignment of ^{13}C resonances in complex organic molecules, while it cannot be emphasized too strongly that an unambiguous assignment of the ^{13}C n.m.r. spectrum is an essential pre-requisite of any biosynthetic study using ^{13}C -enriched precursors. This often requires the expenditure of considerable effort."

In austin, the amount of functionality, the large number of quaternary centers and methyl groups, and the unique steric interactions make the assignment of resonances an interesting problem.

The broadband proton decoupled spectrum of austin published by Chexal, et al., is shown in Figure 1. In the spectrum, all signals appear as singlets since ^{13}C - ^1H coupling is eliminated by continuous irradiation of the sample with a second radiofrequency at the proton region, while doing the pulsed irradiation at the carbon frequencies. The proton irradiation also results in increased signal intensities in the nmr spectrum due to the induction of a nuclear Overhauser

Figure 1. Broadband decoupled ^{13}C -nmr spectrum of austin published by Chexal et al. The spectrum was taken in CDCl_3 on a Bruker HFX-90 spectrometer.

effect (42). Resonances for all 27 carbons can be seen in the spectrum, and can be divided into four categories based on their chemical shifts (27).

Assignment of the carbonyl carbons

The assignment of the four carbonyl carbons was considered first. Carbonyl carbons are known to resonate at low fields in the ^{13}C -nmr spectrum, generally between 150 and 220 ppm downfield from TMS. Ester carbonyls usually appear in the range 160-180 ppm. Both the nature of the R and R' groups in the RCOOR' moiety can affect the carbon resonance frequency, although the substitution α to the carbonyl has the greater effect (43). An upfield shift is usually observed for carbonyl carbons in conjugated systems. This effect can be explained in terms of a decreased electron deficiency at the carbonyl carbon due to electron donation by the π system of the double bond (44).

A study of ^{13}C chemical shifts of δ -lactones in steroidal and terpenoid molecules was found in the literature (45). Compounds 20 and 21 were of particular interest, since the substitution of the unsaturated lactones is similar to that of austin. Compound 20 showed a carbonyl resonance of 163.7 ppm, and compound 21, 163.9 ppm. This compares very well with the 163.8 signal in the spectrum of austin. Since all other carbonyl resonances of austin appear about 5 ppm downfield of

this, there is little chance that they could represent the conjugated carbonyl.

The chemical shift of acetate carbonyls can vary slightly. 4-Alkylcyclohexyl and steroidal acetates appear at 170 ± 1 ppm, while 1-methylcyclohexyl acetate is seen at about 168.8 ppm. Isopropyl acetate also is shielded compared with other alkyl acetates, by about 2 ppm (46). Since the remaining carbonyl resonances in austin are 171.0, 170.1, and 168.5 ppm, no assignment of the acetate carbonyl was possible from this data.

A sample of austin was deacetylated using sodium carbonate in methanol, in order to observe which signals would be missing in the spectrum of the diol derivative (22). This compound was found to be highly insoluble in most solvents, and no ^{13}C -nmr spectrum of it could be taken. An oxidation of deacetylaustin (22) was then performed, in an attempt to make a less polar, more soluble compound. Although an MnO_2 oxidation was not successful, pyridinium chlorochromate cleanly produced the 17-one austin derivative (23). Spectral data of this compound are shown in Figures 2, 3, and 4. The ^{13}C -nmr displays a new carbonyl resonance at 194.8 ppm, due to the

Figure 2. Proton nmr spectrum of deacetylaustin-17-one (23). The spectrum was taken in CDCl_3 on a Varian HA-100 spectrometer. The scan width was 1000 Hz and TMS was used as an internal standard.

Figure 3. Infrared spectrum of deacetylaustin-17-one (KBr).

Figure 4. Broadband decoupled ^{13}C -nmr spectrum of deacetyl-17-one. The spectrum was run in CDCl_3 on a Bruker HX 90 spectrometer.

4XP

AUSTIN DERIVATIVE BROADBAND DECOUPLE B50601 14SEPT

NO.	CURSOR	FREQ.	PPM	INTENS.	AREA
1	3125	4409.297	194.791	52544	113600
2	3684	3858.996	170.480	51264	115904
3	3844	3701.486	163.522	65472	121664
4	4185	3365.793	148.692	66176	161984
5	4258	3293.929	145.517	124032	399936
6	4384	3169.890	140.037	56256	111040
7	4443	3111.808	137.471	79552	222720
8	4829	2731.815	120.684	142016	447488
9	4863	2698.344	119.205	115840	472000
10	5611	1961.984	86.675	84800	260224
11	5628	1945.249	85.935	107264	325184
12	5749	1826.132	80.673	97792	280384
13	5787	1788.723	79.021	137600	610752
14	5801	1774.941	78.412	426560	2667136
15	5833	1743.439	77.020	481728	3090262
16	5865	1711.937	75.628	425984	2573760
17	6174	1407.746	62.190	62976	177472
18	6548	1039.566	45.925	89792	310272
19	6655	934.231	41.271	89536	275456
20	6990	604.444	26.702	93568	578624
21	7009	585.740	25.876	84992	517504
22	7028	567.036	25.050	86656	485760
23	7054	541.440	23.919	83840	350976
24	7080	515.845	22.788	84800	411520
25	7187	410.510	18.135	78976	359744
26	7218	379.993	16.787	77568	374976
27	7339	260.876	11.524	81216	362442
28	7604	0.000	0.000	129024	440688

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α,β -unsaturated ketone. The remaining carbonyl signals are at 163.5 ppm, and approximately 170.4 and 170.1 ppm. The latter two values were estimated, since the two peaks were not resolved by the computer. (Figure 5) Thus it appears that the acetate carbonyl of austin resonates at 168.5 ppm, since this peak was not present in the spectrum of the ketone. The other signals are shifted slightly, less than 0.5 ppm, and it is unlikely that this shift is significant in the assignment of the acetate. Differentiating the remaining two carbonyl signals in the spectrum of austin is very difficult due to the proximity of their chemical shifts, and no assignment of these is possible at this time.

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NO.	CURSOR	FREQ.	PPM	INTENS.	AREA
1	3125	4409.297	194.791	52544	113600
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6	4384	3169.890	140.037	56256	111040
7	4443	3111.808	137.471	79552	222720
8	4529	2731.815	120.684	142016	447488
9	4863	2693.344	119.205	115840	472000

Figure 5. Expansion of low field portion of broadband decoupled ^{13}C -nmr spectrum of deacetylaustin-17-one.

Multiplicity of the carbon signals

To facilitate the assignment of the remaining carbons of austin, it was desirable to obtain the ^{13}C - ^1H coupling information lost by broadband proton decoupling. Methine, methylene, and methyl carbons can be distinguished from this data. The technique of off-resonance decoupling, where the sample is irradiated with a second radiofrequency a few hundred Hertz outside of the proton range, provides this short-range coupling information (47). Another technique for observing coupling is the gated decoupling procedure (48). In this technique, the proton region is irradiated, but only for about 1 second. After a 0.5 second delay, the sample is irradiated with the pulse at the ^{13}C frequencies, and normal pulsed Fourier transform methods are carried out. Since coupling returns immediately after irradiation, but normal nuclear energy level populations do not, the nmr spectrum will display ^{13}C - ^1H coupling, without the total loss of nuclear Overhauser effect. This technique was used on a sample of austin. Approximately 200 mg of sample was required, due to decreased signal intensities as compared with broadband decoupling. Austin for this experiment was obtained by growing flasks of A. ustus on autoclaved black-eyed peas. The austin was then purified by column chromatography, thin layer chromatography, and recrystallization.

In the gated decoupled spectrum of austin, the following multiplicities were observed: 171.0 (s), 170.1 (s), 168.5 (s), 163.8 (s), 146.7 (d), 143.8 (s), 137.6 (s), 132.7 (s), 120.2 (d?), 118.2 (m), 85.6 (s), 84.2 (s), 80.7 (s), 78.9 (d?), 74.8 (d?), 63.0 (s), 46.7 (s) and 42.2 (s). Although there was no difficulty in determining singlet peaks in the spectrum, the multiplicity of the other signals was sometimes ambiguous due to the overlapping of signals. The splitting of the rest of the carbons of austin was indiscernible.

Assignment of the olefinic carbons

There are six olefinic carbons in austin. The trends expected in the chemical shifts of the conjugated double bond carbons can be observed in the model compounds 20 and 21. The olefinic carbons α to the carbonyl in these model compounds resonate at 121.6 and 122.2 ppm respectively. In contrast, the β carbons appear downfield, both at 145.4 ppm. In an exocyclic double bond, the methylene carbon characteristically resonates at high fields, while the olefinic ring carbon comes at rather low fields. This pattern is exemplified in compounds 24, 25 and 26 (49). Utilizing the information in the gated decoupled spectrum, it appears, then, that the 146.7 and 143.8 ppm signals of austin represent the β carbon of the unsaturated lactone and the ring carbon of the exocyclic double bond, respectively; the 137.6 and 132.7 ppm signals are from the tetrasubstituted double bond; and the 120.2 and

24

25

26

118.2 ppm signals are due to the α carbon of the unsaturated lactone and the methylene carbon of the exocyclic double bond. The multiplicity of the signals in the gated decoupled spectrum was not clear enough to assign these last two signals with certainty.

To confirm this assignment, a single frequency proton decoupling experiment was performed. During the pulsed Fourier transform ^{13}C -nmr, the sample of austin was irradiated at the proton frequency corresponding to δ 5.6 ppm. One-half watt decoupling power was used. In the proton spectrum, δ 5.6 ppm represents the approximate midpoint between the two geminally coupled signals of the exocyclic double bond methylene protons. The resulting spectrum is shown in Figure 6. The carbon signal at 117.7 ppm (118.2 in previous spectrum) is a singlet, while the 120.2 signal is split into a doublet. Therefore, the 118.2 peak can be assigned to carbon 29, and carbon 2 resonates at 120.2 ppm.

A positive assignment of the carbons of the tetrasubstituted double bond cannot be made without more information. It is likely that the 137.6 ppm signal represents carbon 18,

Figure 6. ^{13}C -nmr spectrum of austin. Single frequency proton decoupled at δ 5.6 ppm.

AUSTIN IN CDCL ₃ SINGLE FREQ. DECOUPLE @5.6PPM						
NO.	CURSOR	FREQ.	PPM	INTENS.	AREA	
1	3676	3865.961	170.788	37344	133760	
2	3706	3836.428	169.483	33504	133984	
3	3733	3809.848	168.309	13072	126208	
4	3833	3711.405	163.960	10064	80432	
5	3841	3703.529	163.612	10176	40256	
6	4163	3386.540	149.608	6224	39376	
7	4288	3263.485	144.172	5184	39152	
8	4316	3235.921	142.954	13760	122752	
9	4450	3104.006	137.127	16704	104352	
10	4557	2998.672	132.473	13712	127696	
11	4792	2767.329	122.253	6736	39200	
12	4897	2663.963	117.687	5568	23120	
13	5553	2018.171	89.157	32656-	66576	
14	5640	1932.525	85.373	15712	125152	
15	5681	1892.163	83.590	7744	89488	
16	5754	1820.299	80.416	20576	170672	
17	5799	1776.000	78.459	27872	112784	
18	5832	1743.513	77.023	33504	217488	
19	5864	1712.011	75.632	26624	109104	
20	5974	1603.723	70.848	5152-	7232	
21	6163	1417.664	62.628	8144	65087	
22	6209	1372.380	60.628	5728-	15584	
23	6539	1047.515	46.276	9488	89984	
24	6641	947.103	41.840	12960	107632	
25	6866	725.604	32.055	6528	197920	
26	6931	661.616	29.228	17600	489232	
27	6992	601.565	26.575	13216	463264	
28	7034	560.219	24.749	10392	96288	
29	7046	548.405	24.227	10864	104336	
30	7062	532.654	23.531	10608	72384	
31	7099	496.230	21.922	15040	224784	
32	7128	467.681	20.660	6720	34128	
33	7172	424.366	18.747	10576	87968	
34	7223	374.160	16.529	9520	40192	
35	7294	304.265	13.441	8416	30272	
36	7312	286.545	12.658	8096	35696	
37	7379	220.587	9.745	6112	23104	

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and the 132.7 ppm signal is from carbon 19. Methyl substitution at carbon 1 in a 1,2-alkene causes a downfield shift at carbon 1. Likewise, methyl substitution at an α carbon causes a downfield shift to the near olefinic carbon (50). This trend is fairly general, and so carbon 18, having one quaternary α carbon and one trisubstituted α carbon, would be expected further downfield than carbon 19, which has one quaternary α carbon and a primary α carbon. It was hoped that the austin ketone derivative 23 would clarify this assignment, since the double bond in question is conjugated to the carbonyl in this compound. However, in the ketone compound, all olefinic chemical shifts are somewhat different than in the parent compound. It is difficult to determine which signals in the parent compound correspond to those in the ketone derivative. Therefore, no assignment was possible from the study of this derivative.

Assignment of the carbons next to oxygens

There are five signals which can be recognized by their chemical shifts to be due to carbons next to oxygens. Two of these were not singlets in the gated decoupled spectrum. For both the signal at 78.9 ppm and that at 74.8 ppm, one side of the doublet was visible, but the other side was obscured by the chloroform signals. The only protonated carbons next to oxygens in austin are carbons 11 and 17. A single frequency decoupling experiment was performed to distinguish the two.

The quartet at δ 4.4 ppm in the proton nmr was irradiated with 0.5 watt decoupling power during the acquisition of the ^{13}C -nmr spectrum. It was not clear in the resulting spectrum, however, which signal remained coupled. The reason for the failure of the experiment is uncertain. It is suspected that either too much decoupling power, or an incorrect frequency setting, caused both peaks to be mostly decoupled. The assignment of the peaks became apparent when the ketone derivative of austin was made. In the spectrum of the derivative, no peak is present near 74.8 ppm, but there is a signal just above the lowest frequency chloroform signal, at 79.0 ppm. The 78.9 ppm signal of austin must represent carbon 11. The 74.8 ppm signal can be correlated to carbon 17, which becomes a carbonyl carbon in the ketone derivative. No individual assignments of the remaining three carbons next to oxygens were made.

Other assignment attempts

From the gated decoupled spectrum of austin it was apparent that the three quaternary carbons of austin, 4, 7, and 8, gave rise to the signals at 63.0, 46.7, and 42.2 ppm. Lanthanide shift reagents have been used as an aid in the assignment of ^{13}C -nmr spectra (51). It was thought that their use might clarify the assignment of the quaternary carbons, as well as other carbons. Increasing amounts of a shift reagent are added to a sample, and the resulting changes in

the chemical shifts of the carbons are noted. The carbons nearest to the coordinated lanthanide reagent show the greatest change in chemical shift. By relating the changes in chemical shift in the proton nmr to those observed in the ^{13}C -nmr, information is obtained which can help correlate the signals of the spectrum to their respective carbons. The success of this experiment partially depends on how well the shift reagent coordinates at one site in the molecule. This method was tested on the austin molecule by adding 0.019 g of $\text{Eu}(\text{fod})_3$ to 45.8 mg of austin in 0.4 ml of CDCl_3 . The proton nmr spectrum was recorded (Figure 7). A comparison of the normal proton spectrum and this spectrum was done. The proton showing the greatest lanthanide induced shift (LIS) 0.73 ppm, was the β olefinic proton of the α,β -unsaturated lactone. The proton on carbon 17 also showed a sizeable shift. The hydroxyl hydrogen, expected to show the greatest LIS, was only 0.31 ppm further downfield. From an examination of the carbon shifts, it appeared that the shift reagent coordinated somewhat with each ester moiety in the molecule. The results were not clear enough to effectively use this method to assign the ^{13}C -nmr spectrum.

Single frequency decoupling experiments were also attempted on the pro-R proton on carbon 6, the vinyl methyl group, and the acetate methyl group. None were successful. The proximity of neighboring signal in the proton nmr pre-

Figure 7. Proton nmr spectrum of austin plus 0.019 g $\text{Eu}(\text{fod})_3$. The spectrum was recorded in CDCl_3 on a Varian HA-100 spectrometer. The sweep width is 1000 Hz and TMS was used as an internal standard.

vented effective single frequency decoupling, and the complexity of the methyl region in the ^{13}C -nmr obscured decoupled signals.

Culture of the Fungus and Feeding Experiments

Obtaining a suitable liquid medium

Before attempting a ^{13}C acetate feeding experiment, some conditions of fungal culture had to be established since little work was previously done in this area. First it was necessary to find a suitable liquid medium on which to grow A. ustus. A liquid medium facilitates an even distribution of a labeled precursor after fungal growth has begun. This can be a factor in securing a good incorporation of precursor, since incorporation is often highest when the precursor is fed at the onset of secondary metabolite production (40).

A liquid medium made from black-eyed peas proved unsatisfactory due to poor fungal growth. When the fungus was grown on Czapek Dox broth, few secondary metabolites appeared to be produced. Austin was possibly produced on modified Fries' medium, a black-eyed pea meal medium, and a glucose-calcium nitrate-potassium nitrate medium. The modified Fries' medium gave evidence of producing more austin and fewer other metabolites. The presence of austin in A. ustus cultures on this medium was confirmed, and approximately 23 mg could be obtained per liter of prepared medium.

Optimization of metabolite yield

Austin production began about four days after inoculation (Figure 8). Peak production occurred from the fifth day until the ninth day. The maximum amount of austin was estimated to be present between the twelfth and fourteenth day. Some variations on the medium were tested for their effect on austin biosynthesis. A higher yield of metabolite was desirable for ^{13}C -nmr biosynthetic studies. A high pressure liquid chromatograph of altered media extracts suggested greater amounts of austin (ca. 4 times as much) were possibly present when A. ustus was grown on the modified Fries' medium with 100% more yeast extract added, when 0.0328 g of sodium acetate was added, or when the fungus was grown under high light intensity at 26°C in a growth chamber. It was also determined that the fungus grew when the medium was 24 mM in sodium acetate, however, toxicity effects in the form of lower austin production could be observed when the medium was 10 mM in acetate.

Further studies showed that another metabolite of similar R_f to austin was produced when the fungus was grown under high light intensity in the growth chamber, however, little or no austin was present. When a flask of fungus was grown in the dark in the growth chamber, austin was made, so light seemed to be a factor affecting secondary metabolism. Higher austin production under the condition of extra yeast extract or the presence of 1 mM sodium acetate in the medium could not be

Figure 8. Amount of austin present in modified Fries' medium as a function of time.

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positively established. Although in repeating the experiment austin was isolated under these conditions, only 7 to 9 mg, the usual amount per flask, were obtained. This does not necessarily contradict the results of the previous experiment however, since all extracts, even the control grown on normal modified Fries' medium, showed a different metabolite profile in the high-pressure liquid chromatogram of the extracts in this experiment. Lack of time prohibited further experiments to determine the other factors critical to high austin yields.

Effect of day of addition on incorporation of ^{14}C -acetate

Prior to feeding ^{13}C -acetate for biosynthetic studies, it is generally recommended that ^{14}C labeling be done to determine the feasibility of the experiment, and the conditions resulting in the least dilution of label (40). It was decided that the feeding experiments would be done on the yeast extract enriched medium, since it gave no less austin and possibly would give more than the normal modified Fries' medium. There was evidence that the maximum amount of austin was present at an earlier day of growth on this medium, so all cultures were harvested on the eleventh day. Ten microcuries of $[1-^{14}\text{C}]$ -acetate were fed to cultures of A.ustus grown on modified Fries' medium plus 100% extra yeast extract on the third, fourth, fifth and sixth day after inoculation. On the eleventh day after inoculation the flasks were har-

vested. Sixteen to 26 mg of austin, approximately twice the usual amount, were obtained from each flask. This may have been due to inoculation with more spores (5 ml of spore suspension) or due to incubation at warmer temperatures (25-28°C). The greatest incorporation (0.1%) occurred when the fungus was fed labeled acetate on the third day of growth (Table 4). No incorporation occurred in the case of the sixth-day feeding.

Table 4. Variation of amount of incorporation with day of addition of labeled acetate.

Flask	Day Fed	Incorporation	Dilution
1	3	0.1%	7300
2	4	0.05%	23000
3	5	0.006%	100000
4	6	0	∞

Effect of amount of labeled acetate fed on dilution of label

In doing ^{13}C -nmr biosynthetic studies, minimum dilution of label is more important than percent incorporation (52). An experiment was done in which various concentrations of sodium acetate, with a tracer of ^{14}C -labeled acetate, were fed to A. ustus. The results are tabulated in Table 5. The lowest dilution factor obtained was 14.

Table 5. Variation of dilution of label with concentration of added acetate and day fed.

Flask	Day fed	Amt. fed	Dil.	Incorp.
5	2	0.0328 g	60	0.1%
6	3	0.0328 g	140	0.05%
7	3	0.1639 g	20	0.07%
8	3	0.3279 g	14	0.02%

Effect of pH on dilution of label and metabolite production

It is known that in some fungi, small organic acids, such as acetic acid, enter the cell most rapidly by diffusion of the undissociated acid (53). If the pH of the medium is more than one unit above the pK of the acid, little or none of the acid may enter the cell. It was of interest to determine if controlling the pH would have a positive effect on incorporation, since the final pH of the medium after A. ustus growth was about 8. Two flasks of fungus, 10 and 11, were fed 1 mM and 5 mM sodium acetate with a ^{14}C tracer, and the flasks were acidified with HCl daily. After 11 days growth, very little austin appeared to be present in flask 10. This may be due to a toxicity effect, since at low pH acetic acid is more toxic to many fungi than at high pH (54). Approximately 3 mg of austin were obtained from flask 11, having a dilution factor greater than 25. This factor was not as low as those obtained

for the austin from flasks 7 and 8. In a study by Moyer and Coghill on the incorporation of an acidic precursor, the pH was raised in the culture to allow the feeding of greater concentrations of the acid with less toxicity. This resulted in a higher incorporation (55). It is not known whether such a pH adjustment would be beneficial in this case. If pH is the problem causing a low incorporation of acetate, feeding mevalonic acid might be advantageous.

Feasibility of ^{13}C -nmr biosynthetic study

At the present time, it is recommended that dilution per labeled site not exceed 100 in ^{13}C -nmr biosynthetic studies. When 90% enriched ^{13}C -precursors are fed to an organism and a dilution per labeled site factor of 100 is obtained, the labeled sites of the metabolite will show approximately 1% ^{13}C enrichment. Thus enriched sites will have almost twice the natural abundance of ^{13}C , and can be unambiguously determined in spite of peak height fluctuations in the ^{13}C -nmr. If austin is a sesterterpene, according to the theory of isoprenoid biosynthesis (56), 11 sites in the austin molecule should be labeled by feeding $[1-^{14}\text{C}]$ -sodium acetate. A dilution factor of 14 would represent a dilution per labeled site factor of 150 in such a molecule (40). If 90% enriched ^{13}C sodium acetate were fed, isotopic enrichments at labeled sites of approximately 0.6% could be expected in austin. The labeled sites are difficult to distinguish with certainty with

this amount of enrichment. In addition, the low yield of metabolite obtained in this feeding (6 mg) would make acquisition of the ^{13}C -nmr very time consuming, and would lessen the possibility of determining enhanced signals in the spectrum. With the present FT-nmr capabilities, a complete ^{13}C -sodium acetate labeling experiment would be very difficult.

Doubly labeled ^{13}C -acetate could probably be fed with good results if sufficient amounts of metabolite were obtained. Since labeled carbons display ^{13}C - ^{13}C coupling when doubly labeled acetate is incorporated, they can be more easily identified by the presence of satellite peaks. Since the natural abundance of the satellite peaks is only 0.012%, this method is more sensitive than feeding singly labeled acetate, and can be used with enrichments as little as 0.2% (57). The best results would probably be obtained if 10 mM acetate were fed on the second day after inoculation of the fungus.

New Metabolites Isolated

A small amount of white solid was isolated from modified Fries' medium after 10 days of A. ustus growth. The metabolite appeared to be identical with synthetic deacetylaustin (22).

From the six-day extract of modified Fries' medium plus 100% extra yeast extract was isolated an alkaloid having a molecular weight of 239. From mass spectral data and nmr data, the molecular formula was found to be $\text{C}_{15}\text{H}_{13}\text{NO}_2$. Spectral

data suggested that the compound had an aromatic nucleus, with three methyl group substituents and a carboxaldehyde group.

The compound was crystallized from methanol for X-ray crystallographic analysis.

CONCLUSIONS

The assignments made in the ^{13}C -nmr spectrum of austin are shown in Figure 9. The lowest dilution factor obtained in $[1-^{14}\text{C}]$ -sodium acetate feeding experiments was 14. This dilution factor is sufficiently low to allow a successful feeding of $[1,2-^{13}\text{C}_2]$ -sodium acetate. A new metabolite of *A. ustus*, deacetylaustin, was isolated and identified. An alkaloid of unknown structure was also isolated from the fungus.

Figure 9. Assignments made in the ^{13}C -nmr spectrum of austin.

EXPERIMENTAL

Equipment and Materials

Melting points were determined on a Kfller hot-stage melting point apparatus and are corrected. Infrared spectra were recorded on a Beckman IR 4250 spectrophotometer using KBr pellets. Proton nuclear magnetic resonance spectra were run on a Varian Model HA-100 or Varian Model EM 360 spectrometer. CW signal averaging was done on the Varian Model HA-100 using a Nicolet 535 Signal Averager. Chemical shifts are reported in parts per million downfield from internal tetramethylsilane. UV spectra were measured on a Cary Model 14 or Cary Model 118 spectrophotometer in 95% ethanol. The mass spectra were obtained from an A.E.I. MS 902 high resolution mass spectrometer. ^{13}C -nmr spectra were run on a Bruker HX 90 with a spectrometer frequency of 22.636 MHz, equipped with a Nicolet 1080 data system. Chloroform- d_1 was used as the solvent, and chemical shift values are given in parts per million from TMS. High pressure liquid chromatography was done on a Waters Associates Model 202 HPLC using a 254 nm detector and a pre-packed μ BONDAPAK C_{18} column. Radioactivity measurements were obtained from a Packard Tri-Carb 2425 liquid scintillation spectrometer.

$[1\text{-}^{14}\text{C}]$ -sodium acetate (2.55 Ci/mole) and calibrated ^{14}C -labeled toluene were purchased from New England Nuclear. Column chromatography was done on silica gel obtained from

the J. T. Baker Chemical Company or Fisher Scientific Company. Thin layer chromatography was done either on glass plates coated with EM silica gel 60 PF-254, or EM Reagents pre-coated silica gel 60 F-254 TLC plates.

Synthetic Procedures

Preparation of deacetylaustin (22)

A small-scale reaction was performed initially on 2 mg of austin. TLC (elution with 25:1 v:v chloroform:methanol) indicated that one product, more polar than austin, was formed. Approximately 75 mg of austin were stirred in 10 ml of 0.5 molar sodium carbonate in methanol. When the reaction was complete, as determined by TLC, the methanol was evaporated under reduced pressure. Water and ethyl acetate (10 ml each) were added to the flask, and after agitation the layers were separated. The water layer was extracted again with ethyl acetate. The combined ethyl acetate solutions were washed with water and evaporated in vacuo to give a white solid. The solid was recrystallized from methanol. mp. 320-322°C (274-276°C decomposition); for ir see Figure 10; due to insolubility, only a weak nmr could be obtained (Figure 11). It was taken in a CD₃OD/CDCl₃ solution using CW signal averaging techniques (44 scans).

Figure 10. Infrared spectrum (KBr) of synthetic deacetylaustin (22).

Figure 11. Proton nmr of deacetylaustin (22). The solvent was a $\text{CD}_3\text{OD}/\text{CDCl}_3$ solution. Forty-four scans were accumulated and averaged on a Varian HA-100 spectrometer. TMS was used as an internal standard.

Attempted oxidation of deacetylaustin (58)

To a stirred mixture of deacetylaustin (3.2 mg) in chloroform (1.2 ml) was added activated Attenburrow manganese dioxide (30 mg). Stirring was continued at room temperature overnight. No reaction was observed by TLC. After refluxing the mixture for another 12 hours, there was still no sign of reaction. Starting material was recovered.

Acetylation of deacetylaustin (59)

To a mixture of deacetylaustin (2 mg) and pyridine (0.2 ml) was added acetic anhydride (0.2 ml). The reaction mixture was stirred overnight at room temperature. The work-up consisted of pouring the reaction mixture over ice and extracting with chloroform. The chloroform layer was separated, washed with water and evaporated under reduced pressure. A TLC of the product showed the appearance of a spot with the R_f of austin. mp of crude product, 277-285°C.

Preparation of deacetylaustin-17-one (23) (60)

The reaction was first performed on 2.9 mg of deacetylaustin. TLC and an ir spectrum suggested that the correct product was formed cleanly. Deacetylaustin (44.8 mg) was stirred with dry CH_2Cl_2 (20 ml), and pyridinium chlorochromate (0.7041 g) was added. Stirring was continued at room temperature for 24 hours. Ether (100 ml) was then added, and the solvent was decanted. The brown solid (reduced reagent) was

washed twice with ether. The combined organic extracts were filtered through a small column of Florisil. After evaporation of the eluent, 17.5 mg of product were obtained. When the column was eluted with 80 ml of chloroform, an additional 17 mg of product were isolated, to give a total yield of 77%. The compound was recrystallized from methanol. mp 244-247°C; for nmr and ir see Figures 2 and 3; uv $\lambda_{\text{max}}^{\text{EtOH}}$ 269 nm (ϵ , 7650); ms m/e 456.1773 (M^+). (Calc. for $C_{25}H_{28}O_8$, 456.1784).

Production of Austin on Various Media

Isolation of austin from *A. ustus* on black-eyed peas

Black-eyed peas (130-135 g of "Brown's best black-eyed beans") were measured into each of 39 Fernbach flasks, and distilled water was added. After soaking the peas for 30-60 minutes, the water was decanted, and the flasks were plugged with cotton and autoclaved (15 lb pressure, 121°C, 15 minutes). Inoculum was prepared from two sporulated *A. ustus* cultures grown on potato dextrose agar in 100 ml Erlenmeyer flasks. Sterile distilled water (approx. 40 ml) and glass beads were added to the sporulated cultures. Agitation then produced a spore suspension. One to two ml of this spore suspension were syringed through the cotton plugs into each of the Fernbach flasks. After four days growth under normal laboratory temperature and lighting conditions, the cultures were thriving and appeared to be sporulated. Eight to ten days after inoculation, the flasks of fungus were harvested. The

moldy pea mats were ground in chloroform in a Waring Blendor, the liquids were strained off, and the solids were reground in more chloroform. The water layer was then removed from the combined chloroform extracts. After drying over anhydrous sodium sulfate, the extracts were filtered through Celite. The chloroform was evaporated under reduced pressure to give 100 g of a brown oil. A thin layer chromatograph of the extract is shown in Figure 12. The oil was partitioned into hexane soluble and hexane insoluble portions. The hexane insoluble extract was column chromatographed in approximately 10 g portions on 350 g of silica gel. Chloroform (ca. 3 l) and 40:1 chloroform:methanol (ca. 3 l) were used as the eluents. The fractions from the column which contained austin according to TLC were evaporated, and re-chromatographed on a 70 g silica gel column. It was eluted with chloroform (500 ml), 60:1 chloroform:methanol (500 ml) and 50:1 chloroform:methanol (700 ml). The fractions from this column which contained austin were chromatographed on preparative layer silica gel plates using 25:1 (v:v) chloroform:methanol as the mobile phase. The uv-absorbing austin bands were scraped from the plates and washed with chloroform and methanol to isolate the purified austin. The austin was recrystallized from methanol and combined with previously isolated austin for ¹³C-nmr studies.

a. austin.

b. CHCl_3 extract of A. ustus grown on black-eyed peas.

Eluent: 25:1 (v:v) chloroform:methanol.

Detection: 254 nm uv light.

Figure 12. Schematic diagram of thin layer chromatogram.

Preparation of black-eyed pea liquid media

Four flasks of medium were prepared by grinding water-softened black-eyed peas in a blender with distilled water. After straining out the solids with cheese cloth, 400 ml of the pea solution were put in each of the Fernbach flasks. Three flasks of medium were prepared in a similar way, only the peas were soaked and ground in a 10% sucrose solution. The pea solution was poured into three flasks (350 ml in each). The pea solids were then put into two more flasks. All flasks were plugged with cotton, and autoclaved at 121°C, 15 lb pressure, for 15 minutes. The media were then inoculated in the manner described earlier. The growth of the fungus and the metabolite production on the pea liquid media were not satisfactory.

Czapek Dox broth

Difco Czapek Dox broth (17 g) was dissolved in distilled water (500 ml). (Composition of broth per liter of medium: 30 g of Difco saccharose, 3 g of sodium nitrate, 1 g of dipotassium phosphate, 0.5 g of magnesium sulfate, 0.5 g of potassium chloride, and 0.01 g of ferrous sulfate.) The medium was divided into two Fernbach flasks. The flasks were plugged with cotton and autoclaved at 240°F, 15-20 lb pressure for 20 minutes.

Modified Fries' medium

Modified Fries' medium (500 ml) was prepared in the following way. Ammonium tartrate (2.5 g), ammonium nitrate (0.5 g), potassium dihydrogen phosphate (0.5 g), magnesium sulfate heptahydrate (0.25 g), sodium chloride (0.05 g), calcium chloride dihydrate (0.065 g), sucrose (15 g), and Difco yeast extract (0.5 g) were weighed out ($\pm$ 0.5 mg). One-half milliliter of an iron sulfate solution (2 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ in 100 ml distilled water) was added, and distilled water was added to make 500 ml of solution. The medium was equally divided into two Fernbach flasks. They were plugged with cotton, and autoclaved for 20 minutes at 236°F and 12-14 lb of pressure.

Black-eyed pea meal medium

Black-eyed pea meal was made by grinding black-eyed peas into a fine powder with a mortar and pestle. Then the black-eyed meal (2.5 g), glucose (10 g), Difco yeast extract (2.5 g), sodium chloride (2.5 g), and dipotassium hydrogen phosphate (2.5 g) were weighed out ($\pm$ 8 mg), and combined in a flask. Distilled water was added to make 500 ml of medium, and the pH was adjusted to 6.4 with HCl. The medium was put into two Fernbach flasks, and cotton plugs were inserted.

A synthetic medium

Calcium nitrate tetrahydrate (405 mg), potassium nitrate (80 mg), potassium chloride (65 mg), magnesium sulfate (360 mg), sodium dihydrogen phosphate monohydrate (22 mg), sodium sulfate (199 mg), ferric chloride hexahydrate (3.4 mg), manganese sulfate monohydrate (4 mg), boric acid (1.3 mg), potassium iodide (0.7 mg), and glucose (30 g) were weighed out (+ 1 mg) and combined. Distilled water was added to make 1000 ml of medium, and the pH was adjusted to about 5.5 using NaOH and HCl. The medium was put into two Fernbach flasks which were then plugged with cotton. This medium and the black-eyed pea meal medium were autoclaved at 20 lb pressure and 121°C for 20 minutes.

Austin production on the four media

The four media were inoculated according to the method previously described. After two days, all cultures were growing, however, the cultures on the modified Fries' medium and black-eyed pea meal medium grew fastest. Eleven days after inoculation, one flask of fungus on each medium was harvested. Each fungal mat was separately ground in a Waring Blendor in chloroform. The extract was then filtered on a Büchner funnel, and the water layer was separated from the organic layer. The chloroform extract was filtered through anhydrous sodium sulfate on Celite, and evaporated in vacuo.

No austin was detected in any of the extracts by TLC using a vanillin/sulfuric acid spray reagent.

The media from the four flasks were extracted by shaking them in a separatory funnel with chloroform. The chloroform layer was separated, dried and filtered, and evaporated. A TLC of the extracts (Figure 13) indicated that austin was possibly present in all but the Czapek Dox medium. It appeared to be present in the greatest proportions in the modified Fries' medium.

Isolation of austin from modified Fries' medium

Five liters of modified Fries' medium were prepared. Four hundred milliliters of the medium were put into each of 11 Fernbach flasks, and 300 ml were put into two flasks. The flasks of medium were autoclaved at 121°C and 20 lb of pressure for 15 minutes. The flasks were inoculated in the usual manner, with 2 ml of a spore suspension made from an A. ustus culture grown on potato dextrose agar in a 250 ml Erlenmeyer flask. The cultures were incubated at room temperature under normal laboratory lighting conditions. Ten days after inoculation, 10 of the cultures were terminated by pouring the medium from the flasks. The medium was extracted with chloroform, filtered, and dried with anhydrous sodium sulfate. After evaporation of the solvent, 0.7161 g of a brown oil was obtained. The extract was column chromatographed on 50 g of silica gel.

a. Austin.

b. CHCl_3 extract of A. ustus on black-eyed pea meal medium.

c. CHCl_3 extract of A. ustus on Czapek Dox broth.

d. CHCl_3 extract of A. ustus on modified Fries' medium.

e. CHCl_3 extract of A. ustus on synthetic medium.

Eluent: 25:1 (v:v) chloroform:methanol.

Detection: Vanillin/ethanol/sulfuric acid spray reagent.

Figure 13. Schematic diagram of thin layer chromatogram.

The column was eluted with chloroform, 100:1 chloroform:methanol, and 50:1 chloroform:methanol. The fractions containing austin were evaporated and 0.088 g of the semi-pure compound was obtained.

Austin production as a function of time

From three of the flasks containing 400 ml of medium, samples (10 ml) were taken of the medium. This was done by inserting a sterile volumetric pipette between the cotton plug and the neck of the flask, and withdrawing the proper amount of liquid from beneath the fungal mat. Twenty-five samples were taken between day one and day 28. First, 9 samples were taken from the first flask, then 9 samples from the second flask, then 7 from the third flask. The samples were stored in the freezer until use.

Each medium sample was shaken fifty times with 10.0 ml of chloroform. The organic layer was separated, filtered, and evaporated under reduced pressure. Methanol (0.50 ml) was then added to the extract, and 5 μ l of this solution was injected into the high pressure liquid chromatograph. 70:30 methanol:water was used as the eluent. The flow rate was 1 ml/minute and the pressure was 2000 psi. The austin peak was identified by a comparison of the retention time of the extract components with the retention time of an authentic austin sample. The amount of austin injected was found to be

approximately proportional to the height of the austin peak on the chart paper.

Modified Fries' medium variations

Potassium dihydrogen phosphate ($8 \text{ g} \pm 0.3 \text{ mg}$), magnesium sulfate heptahydrate ($4 \text{ g} \pm 0.1 \text{ mg}$), sodium chloride ($0.8 \text{ g} \pm 0.1 \text{ mg}$), calcium chloride dihydrate ($1.04 \text{ g} \pm 2 \text{ mg}$), and sucrose ($240 \text{ g} \pm 0.01 \text{ g}$) were weighed out and combined in a large bottle. Eight milliliters of an iron sulfate solution ($2 \text{ g FeSO}_4 \cdot 7\text{H}_2\text{O}$, distilled water added to make 100 ml) was added. Distilled water was then added to make 8 l of solution. Four hundred ml of this solution was added to flasks 1 through 10, which contained the nitrogen sources and yeast extract shown in Table 6. To the remaining 4 l of solution were added ammonium nitrate ($4 \text{ g} \pm 0.3 \text{ mg}$), ammonium tartrate ($20 \text{ g} \pm 0.1 \text{ mg}$), and yeast extract ($4 \text{ g} \pm 0.5 \text{ mg}$). Four hundred ml of this normal modified Fries' medium were put into flasks 11 through 16. Sodium acetate (0.0328 g, 0.328 g, 0.800 g) was added to flasks 11, 12, and 13, respectively. Flask 14 was used as a control. Flask 15 was grown at 26°C in a growth chamber with high light intensity (ca. 1200 ft-candles). Flask 16 was used as a control and was not inoculated.

All but flask 16 were inoculated with 2 ml of a spore solution made by adding 60 ml of sterile distilled water to a 34 day old A. ustus culture grown in a 250 ml Erlenmeyer flask. Four 10 ml samples of the media were taken from each

Table 6. Nitrogen sources and yeast extract in flasks 1 through 10.

Flask No.	Nitrogen Sources	Amount of yeast Extract
1	0.53 g ammonium nitrate 2 g ammonium tartrate	0.4 g yeast ex.
2	2 g ammonium tartrate	0.4 g yeast ex.
3	0.4 g ammonium nitrate 1.152 g ammonium chloride	0.4 g yeast ex.
4	0.4 g ammonium nitrate	0.4 g yeast ex.
5	2.68 g sodium nitrate	0.4 g yeast ex.
6	1.2 g sodium nitrate	0.4 g yeast ex.
7	4 g ammonium tartrate 0.4 g ammonium nitrate	0.4 g yeast ex.
8	0.8 g ammonium nitrate 2 g ammonium tartrate	0.4 g yeast ex.
9	0.4 g ammonium nitrate 2 g ammonium tartrate	No yeast ex.
10	0.4 g ammonium nitrate 2 g ammonium tartrate	0.8 g yeast ex.

flask between day 6 and day 14. All samples were stored in the freezer until the end of the sampling period. Then a sample from each flask was extracted with chloroform. The chloroform and water layers were separated, and the chloroform extract was filtered (multiple times if necessary to clear up emulsions) and evaporated. One-half ml of methanol was added to the extract, and 1-5 μ l of this solution was

injected into the HPLC. The eluent was 70:30 methanol:water, the pressure was maintained at 1500-2000 psi, and the flow rate was 1 ml/minute. All media extract samples were chromatographed for flasks which appeared to contain austin.

Optimization of growth conditions

Twelve liters of modified Fries' medium were prepared. Four hundred ml of the medium were put into each of 10 Fernbach flasks. Thirty-six hundred ml were poured into a large flask, and more yeast extract (3.6003 g) was added. This enriched medium was put into nine Fernbach flasks (400 ml in each). Another 3600 ml of medium was put in a separate flask, and sodium acetate (0.2952 ± 0.5 ml) was added. This medium was divided among another nine Fernbach flasks. To the remaining 800 ml of the modified Fries' medium was added yeast extract ($0.8000 \text{ g} \pm 1 \text{ mg}$) and sodium acetate ($0.0656 \text{ g} \pm 0.5 \text{ mg}$). This medium was put into two Fernbach flasks. All flasks were plugged with cotton and autoclaved. They were then inoculated in the usual manner by adding 50 ml of sterile distilled water to a culture of A. ustus grown in a 250 ml Erlenmeyer flask for two weeks. Flasks 1-7, containing normal modified Fries' medium were incubated in the growth chamber at approximately 26°C . Flask 8 was covered with foil, and incubated in the growth chamber. Flasks 9 and 10 were grown as usual on the desktop. Flasks 11-18, containing 0.0328 g of sodium acetate per flask were grown on the laboratory desktop.

Flask 19 containing the same medium was grown in the growth chamber. One flask (Flask 21) of the medium containing both extra yeast extract and sodium acetate was grown in the growth chamber, while the other (Flask 20) was grown on the desktop. Flasks 22-29, containing 100% more yeast extract, were grown on the desktop. Flask 30, containing the same medium, was grown in the growth chamber.

Four samples (5 ml each) were taken from each category of fungal growth conditions between the seventh and eleventh day of growth. Flask 21, containing extra yeast extract, sodium acetate, and incubated in the growth chamber, was not initially sampled due to poor growth.

On the tenth day after inoculation, the medium was poured from flasks 22-29, and from flask 30. On the eleventh day of growth, the medium was poured from flasks 11-18 and 19, and on the twelfth day after inoculation, flasks 1-7 and 8 were harvested. Each type of medium was extracted by shaking it with chloroform. The chloroform layer was filtered through Celite, dried with anhydrous sodium sulfate, and evaporated. A TLC of some of the extracts is shown in Figure 14.

The extract from flask 8 (32 mg) was chromatographed on a column of 12 g of silica gel. The column was eluted with chloroform. Austin, with some impurities (9.3 mg) was obtained as the second major component eluted from the column.

a. Austin.

b. Deacetyl austin.

c. Tenth day extract of *A. ustus* grown on modified Fries' medium plus 100% more yeast extract (Flasks 22-29).

d. Twelfth day extract of *A. ustus* grown on modified Fries' medium in the dark in the growth chamber (Flask 8).

e. Twelfth day extract on *A. ustus* grown on modified Fries' medium in the growth chamber (Flasks 1-7).

Eluent: 25: (v:v) chloroform:methanol.

Detection: 254 nm uv light.

Figure 14. Schematic diagram of thin layer chromatogram.

The extract of flasks 22-29 (160 mg) was column chromatographed on 15 g of silica gel. Elution with chloroform, and 100:1 chloroform:methanol produced 0.053 g of rather pure austin.

The 5 ml samples of media taken from the flasks were extracted in the usual way, and 0.5 ml of methanol was added to each. Five μ l of each methanol solution were injected into the HPLC. The eluent was 70:30 methanol:water. The flow rate was 0.8 ml/min and the pressure was between 2100 and 2500 psi.

Feeding Experiments

Radioassay

Liquid scintillation counting was done in a Bray's solution of the following composition: 60 g naphthalene; 4 g 2,5-diphenyloxazole (PPO); 100 mg 1,4-bis-2(5-phenyloxazolyl)-benzene (POPOP); 20 ml ethylene glycol; 100 ml methanol; dry, peroxide-free dioxane to make one liter. Each sample of austin to be counted (ca. 1 mg) was weighed on a microanalytical balance. The samples were dissolved in enough methanol to make 5 ml of solution. Three aliquots of this methanol solution (0.50 ml each) were counted in 15 ml of the Bray's solution. Counting efficiencies were determined by the internal standard method using calibrated ^{14}C -labeled toluene. They varied from 68% to 73%. Percent incorporation was calculated using the following formula:

$$\% \text{ incorporation} = \frac{\text{total activity of austin}}{\text{total activity fed}} \times 100$$

Isotopic dilution was determined by dividing the specific activity of the precursor by the specific activity of austin. Other radiotracer calculations were done according to the methods outlined by Kobayashi and Maudsley, and those of Wang, Willis, and Loveland (61, 62).

Variation of incorporation with day of addition of ^{14}C -acetate

Four flasks containing modified Fries' medium (400 ml each) enriched with 100% more yeast extract were prepared. The flasks were plugged with cotton and the plugs were covered with foil. The flasks were then autoclaved. A spore solution was made in the usual way by adding sterile distilled water (30 ml) to a sporulated culture of A. ustus grown on potato dextrose agar in a 125 ml Erlenmeyer flask. Five ml of the spore suspension were syringed through the cotton plug into each of the 4 Fernbach flasks. The flasks were incubated in a warm room (25-28°C) under normal laboratory lighting conditions.

Ten milliliters of acetate solution were made by dissolving 250 μCi of $[1-^{14}\text{C}]$ -sodium acetate (8.1 mg) in 95% ethanol. Two milliliters of this solution were pipetted into a volumetric flask, and sterile distilled water was added to make 25.0 ml of solution. Five milliliters of this solution (10 μCi) were pipetted under the fungal mat of one of the

three-day old cultures of A. ustus. The flask was gently swirled to distribute the label. The three remaining flasks of fungus were fed ^{14}C -sodium acetate in the same way on the fourth, fifth, and sixth days, respectively. On the 11th day after inoculation, the brown medium was poured from each of the flasks. Each medium was extracted by shaking it with 350 ml of chloroform, drawing off the chloroform layer, and shaking with another 200 ml of chloroform. The combined chloroform extracts were filtered to eliminate emulsions, and the water layer was removed. Then each extract was dried with anhydrous sodium sulfate. After filtering the extract through a piece of cotton, the chloroform was evaporated in vacuo. The composition of the extracts appeared to be nearly identical by thin-layer chromatography (Figure 15). Each extract was column chromatographed on approximately 11 g of silica gel. Elution was done with chloroform, and chloroform:methanol solutions. The fractions containing austin were evaporated to dryness. A proton nmr of one of the samples confirmed that it was austin. The samples were recrystallized from methanol and counted to a constant activity. The amounts obtained and radioactivity measurements are tabulated in Table 7.

- a. Austin.
- b. Radioactive extract of the flask of *A. ustus* fed a tracer of ^{14}C -acetate on the third day of growth.
- c. Radioactive extract of the flask of *A. ustus* fed 0.3279 g of ^{14}C -acetate on the third day of growth.

Figure 15. Schematic diagram of thin layer chromatogram.

Table 7. Amount and activity of austin isolated from A. ustus cultures fed $[1-^{14}\text{C}]$ -sodium acetate.

Flask	Day Fed	Amt. Extract	Amt. Austin	Specific Activity ^a
1	3	43 mg	17 mg	703,000
2	4	58 mg	26 mg	230,000
3	5	57 mg	16 mg	40,000
4	6	27 mg	16 mg	0

^aSpecific activity of austin is given in disintegrations per minute per millimole.

Variation of dilution with amount of added ^{14}C -acetate

Four flasks of modified Fries' medium were prepared in the usual manner and autoclaved. Each was inoculated with 5 ml of a spore suspension made with 40 ml of distilled water from an A. ustus culture grown in a 250 ml flask. Four sodium acetate solutions were made up. Two contained 0.0328 g of sodium acetate in 5 ml of distilled water. Another contained 0.1639 g of sodium acetate in the same amount of water, and the last contained 0.3279 g of sodium acetate. The solutions were autoclaved, and to each was added 20 μCi of $[1-^{14}\text{C}]$ -sodium acetate (0.8 ml of the previously made 95% ethanol solution). On the second day of growth, one of the 0.0328 g sodium acetate solutions was pipetted into flask 5. On the

third day of growth, the other 0.0328 g acetate solution, the 0.1639 g solution, and the 0.3279 g solution were pipetted into flasks 6, 7, and 8, respectively. On the eleventh day after inoculation, the medium was removed from the flasks. The medium from each flask was extracted with 300 ml of chloroform, then with another 100 ml of chloroform. The extracts were filtered, dried, and evaporated. Each was column chromatographed on about 11 g of silica gel. The samples were recrystallized from CH_3OH and counted to a constant activity. The amounts of austin obtained and its activity are shown in Table 8.

Table 8. Amount and activity of austin isolated from *A. ustus* cultures fed various concentrations of acetate using a ^{14}C tracer.

Flask	Day Fed	Amt. Fed	Amt. Austin	Specific Activity ^a
5	2	.0328 g	15 mg	1760000
6	3	.0328 g	13 mg	740000
7	3	.1639 g	14 mg	1100000
8	3	.3279 g	6 mg	721000

^aSpecific activity is given in dpm/mmole.

Effect of acidification on dilution of label

Modified Fries' medium plus 100% extra yeast extract was prepared for 2 flasks. The medium was autoclaved and inoculated with 4.2 ml of a spore suspension made by adding 15 ml of water to an A. ustus culture grown in a 125 ml flask. Three days after inoculation, one flask (#10) was fed 0.0326 g of sodium acetate plus 20 μ Ci of $[1-^{14}\text{C}]$ -sodium acetate. The other flask (#11) was fed 0.1642 g of sodium acetate plus 20 μ Ci of ^{14}C -labeled sodium acetate. At this time, the pH of the medium was approximately 4. On the fourth day after inoculation, the pH of the flasks was still 4. On the fifth day, the pH had risen to about 5, and a small amount of HCl was added to each flask to bring the pH between 4 and 5. From the sixth day until harvesting, the pH continued to rise, and at least once daily the flasks were acidified to a pH of 5. The medium was poured from the flasks on the eleventh day after inoculation. The medium of each flask was extracted with 300 ml of chloroform, and then extracted again with 100 ml of chloroform. The medium of flask 10, which was kept more acidic, had a golden color, in contrast to the brown medium of the other flasks. After filtering, drying, and evaporating the extracts, thin layer chromatogram of each was done. Flask 10 appeared to contain very little austin. Flask 11 appeared to contain somewhat more. The extract of flask 11 was column chromatographed on 11 g of silica gel. Approximately 3 mg of austin was isolated. It was recrystallized from methanol and

counted to a constant activity. The specific activity was determined to be 775,000 dpm/mmol.

Isolation of New Metabolites

Isolation of deacetylaustin

As mentioned earlier, austin was isolated by column chromatography from the extract of 10 flasks of modified Fries' medium after A. ustus growth. Further elution of the column with chloroform:methanol solutions gave a small amount of white solid (272-295°C decomp.). The R_f of the material was 0.20 (26:1 chloroform:methanol). It gave a color similar to that produced by austin when sprayed with a vanillin/sulfuric acid spray reagent. An ir spectrum of the material showed absorptions at 3480, 1778, 1725, and 1686 cm^{-1} . The ir was nearly identical to synthetic deacetylaustin.

Isolation of an unknown alkaloid

HPLC evidence suggested larger amounts of deacetylaustin were present in A. ustus cultures grown on modified Fries' medium plus 100% extra yeast extract on the sixth day of growth. Twenty flasks of this medium were prepared, autoclaved, and inoculated with 1 milliliter of spore suspension. Six days later the medium was poured from the flasks. The medium was extracted with ethyl acetate (ca. 8 l). The extract was filtered, dried, and evaporated. Approximately

0.85 g of a golden oil remained. A chromatogram of the extract showed the presence of a spot with an R_f near that of deacetylaustin, however, its color was somewhat different when sprayed with the vanillin spray reagent. The extract was column chromatographed, however, no deacetylaustin was isolated. A yellow substance crystallized from some of the early fractions from the column. The compound (mp 171-172.3°C) was slightly less polar than austin by TLC, and looked purple under uv light. It gave a positive test with silicotungstic acid, indicating the presence of a basic tertiary nitrogen. The mass spectrum of the compound showed a molecular ion at m/e 239.0950 (100%), consistent with the formula $C_{15}H_{13}NO_2$. Other peaks include m/e 240 (17.6%), 238 (3.9%), and 224 (6.2%). The pmr and ir of the compound are shown in Figures 16 and 17. UV maxima were observed at 273 (ϵ , 28,200), 297 (ϵ , 26,800), shoulder at 309 (ϵ , 25,800), and 368 nm (ϵ , 9280).

The metabolite was also isolated from flasks (#1-7) grown on modified Fries' medium in the growth chamber for 12 days. The compound was recrystallized from methanol for x-ray crystallographic analysis.

Figure 16. Proton nmr of alkaloid of unknown structure. The spectrum was recorded on a Varian HA-100 spectrometer, with a sweep width of 1000 Hz. The solvent was CCl_4 and TMS was used as an internal standard.

Figure 17. Infrared spectrum (KBr) of alkaloid of unknown structure.

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