METABOLIC PRODUCTS OF MICROORGANISMS 123* THRAUSTOMYCIN, A NEW ANTIFUNGAL NUCLEOSIDE ANTIBIOTIC FROM *STREPTOMYCES EXFOLIATUS*

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A new nucleoside antibiotic, inhibiting the growth of fungi, could be isolated from *Streptomyces exfoliatus*. The antibiotic was found by a screening method based on the inhibition of zygospore formation of *Mucor hiemalis*. It is composed of equal amounts of adenine, L-leucine, and an unusual tetrahydroxy-monocarbonic acid, $C_{11}H_{14}O_6$, with properties similar to a carbohydrate.

In spite of the large number of known antifungal antibiotics, there is continuous interest in new antibiotics with antifungal action. The majority of antifungal antibiotics are polyenes. It is difficult to detect antifungal substances different from polyenes. The test introduced by us to the screening of antibiotics, is based on morphological changes of the hyphae of *Botrytis cinerea* and also allows the detection of antibiotics in addition to polyenes[1]. The sensitivity of the test, however, is limited to antibiotics with strong action against *Botrytis cinerea*.

The test with the zygomycete *Mucor hiemalis* described in this paper, also allows the detection of antifungal action in the presence of polyenes. Furthermore, the inhibition of the growth of the mycelium and the inhibition of the formation of zygospores can be distinguished[2]. With the aid of this test we found a new antibiotic which, because of its instability, we named thraustomycin.

Materials and Methods

1. Assay

a) Preparation of the Spore-Solution

Cultures of *Mucor hiemalis* (+) CBS and *Mucor hiemalis* (−) CBS BB 157 were cultivated for 5~10 days in 500 ml Erlenmeyer flasks with 100 ml yeast-malt-agar (0.4 % yeast extract, 1 % malt extract, 0.4 % glucose, 2 % agar). During this time sporangia were produced in high yield. The spores were suspended in 50 ml 1 % Tween, mixed, divided into aliquots and stored at −18°C. At this temperature the sporangiospores remained viable for 3~4 weeks.

b) Preparation of the Test Plates

2×10^5 Spores were added to 100 ml yeast-malt-agar, and 17.5 ml of this suspension were poured into Petri-dishes. The prepared plates could be stored for 2 days at 4°C. After 3-day incubation in the dark at 18~22°C the zygospores were formed. They were macroscopically detectable as dark points. Inhibition zones, caused by antibiotics in culture brews, were measured in mm.

2. Description of the Strain

The actinomycetales strain Tü 472 was isolated from a soil sample from Bobo-Oncho, Upper

* Metabolic products of microorganisms, 122; R. KANN, E.U. KLEIN-VOGLER and H. ZÄHNER: Über Beziehungen zwischen Makrotetrolidbildung und Kaliumtransport bei *Streptomyces griseus*. Arch. Mikrobiol.

Volta. The strain Tü 472 shows all species-determining characteristics of *Streptomyces exfoliatus* (WAKSMAN *et* CURTIS) WAKSMAN *et* HENRICI[3] and, therefore, it has to be classified under this species.

a) The spores are ellipsoidal (0.5 $\mu \times 0.9\ \mu$) with a smooth surface.

b) The aerial mycelium of spored cultures is of light cinnamon-color (cinnamomeus).

c) The spore-chains are monopodially branched, straight or curved (rectus-flexibilis).

d) On peptone-iron-agar no black pigments were formed.

e) Depending on culture-medium and age, the vegetative mycelium is light- to dark-brown.

The strain Tü 472 exists in two variations. One variation (D) on yeast-malt-agar excretes a brown-black pigment. This variation shows a strong formation of aerial mycelium. During fermentation the pH of the culture-solution reaches a minimum after 50 hours and finally rises to 8. It produces thraustomycin. The other variation (H) produces neither pigments nor antibiotics and shows little formation of aerial mycelium. During the first 120 hours of fermentation the pH stays at 4. For the preparation of thraustomycin seed-cultures were used which had already reached pH 5 and where, therefore, the presence of variation D was certain.

3. Fermentation and Isolation

The strain Tü 472 was incubated in submerged culture at 27°C in 10 liters of culture-medium (2 % soybean meal, 2 % glucose) under stirring (220 rpm) and aeration (2-liter air per minute). The medium was inoculated with 10 % inoculum. As soon as the culture broth had reached pH 6.5 (after 3~4 days), it was brought to pH 4 and the antibiotic was isolated according to the following scheme (Scheme 1). The solution contained 50~100 μg/ml thraustomycin.

Scheme 1. Preparation of thraustomycin. Yield in % (in relation to the activity of the culture broth)

100 % Culture broth
↓
75 % Filtrate
- 2 % charcoal
- 80 % acetone + 0.5 N formic acid

50 % Eluate
- concentration
- countercurrent distribution (70 steps, aqu. buffer, pH 5/butanol)
- evaporation

42 % Raw material

The raw product was further purified by ion-exchange chromatography on SP-Sephadex C 25 (column: 100×1.7 cm) with 0.1 N triethylammonium acetate (pH 4.6) as the elution buffer. The active material was detected in the fraction of 510 to 590 ml.

Yield: 50 % (based on the raw product).

The lyophilized product was put on a column with Sephadex G 25 (superfine; column: 100×3.0 cm) and eluted with 0.01 N acetic acid. Thraustomycin was detected in the fraction of 420 to 470 ml.

Yield: 35 % (based on the raw product).

In addition to thraustomycin another substance was isolated which was found to be a decomposition product of thraustomycin (β-thraustomycin).

4. Formation of Derivatives

a) Hydrolysis: Thraustomycin and β-thraustomycin were hydrolysed in 3 N hydrochloric acid for 2 hours at 100°C.

b) Trimethylsilylation: TMS and TMS-d_9 derivatives were prepared according to standard methods with bis-trimethylsilyl-trifluoroacetamide (Regis Chemical Comp.) and d_9-bis-trimethylsilyl-acetamide (Sharp and Dohme GmbH) respectively.

c) Esterification: Methyl esters were prepared by reaction of the hydrolysate with a solution of hydrogen chloride in dry methanol or CD_3OD respectively at 100°C for 1 hour.

d) Trifluoroacetylation: The TFA group was introduced with trifluoroacetic anhydride in dichloromethane (1 : 4, vol/vol) at room temperature (1 hour).

e) Gas Chromatography-Mass Spectrometry: An LKB 9000 GC-MS combination was used for GC-MS investigations with a 3 m glass column packed with 3 % OV 17 on Chromosorb WAW (100~120 mesh). Mass spectra were taken at 70 eV ionizing energy; accelerating voltage 3.5 kV, ion source temperature 250°C.

f) High Resolution Mass Spectrometry: For exact mass determinations an MS9 instrument (AEI) was used. The peak matching method was applied with heptacosafluorotributylamine as internal standard.

g) Determination of Configuration of Amino Acids: The method described by KOENIG *et al.*[5] was applied for determination of the configuration of the leucine moiety in the antibiotic.

Results

Biological Properties of Thraustomycin

The minimal inhibitory concentration (MIC) of thraustomycin for *Mucor hiemalis* is dependent on the strain and the moment at which the antibiotic is added. The minimal inhibitory concentration in liquid culture is about 2 μg/ml when tested by tube-dilution method in synthetic medium. Thraustomycin is added at the moment of inoculation. This value increases strongly after germination of the spores. The investigation of the spore germination in the microscopic test showed that 0.83 μg/ml thraustomycin inhibited the germination of the (+)-strain by 90%, whereas the same concentration of the antibiotic caused only 30% inhibition of the (−)-strain.

In the plate diffusion test thraustomycin shows no action against bacteria. Against fungi, it shows a relatively narrow action spectrum (Table 1).

Table 1. Minimal inhibitory concentration (MIC) of thraustomycin in the plate diffusion test

Strain	MIC (mg/ml)
A. Bacteria	
Escherichia coli	>100
*Escherichia coli**	>100
Bacillus subtilis	>100
*Bacillus subtilis**	>100
Pseudomonas saccharophila	>100
Streptomyces viridochromogenes	10~30
B. Fungi	
a) Zygomycetes	
Blakeslea trispora (+)	3~ 10
Blakeslea trispora (−)	3~ 10
Mucor hiemalis (+)	0.1~0.3
Mucor hiemalis (−)	30~100
Mucor mucedo (+)	0.1~0.3
Mucor mucedo (−)	0.1~0.3
Mucor luteus (+)	10~30
Mucor luteus (−)	1~ 4
Mucor parvisporus	30~100
Mucor racemosus (+)	10~ 30
Mucor racemosus (−)	10~ 30
Phycomyces blakesleeanus (+)	10~ 30
Phycomyces blakesleeanus (−)	10~ 30
Rhizopus circinans (+)	10~ 30
Rhizopus circinans (−)	10~ 30
Zygorhynchus moelleri	30
b) Ascomycetes	
Aspergillus melleus	>100
Botrytis cinerea	30~100
Candida vulgaris	>100
Paecilomyces varioti	>100
Piricularia oryzae	10~30
Saccharomyces cerevisiae	3
Spicaria sp.	10

* Synthetic medium

Chemical Properties and Structure of Thraustomycin

Thraustomycin is a white solid substance which upon heating decomposes without melting. The UV-spectrum of the aqueous solution is similar to that of adenine. (λ_{max} 259 nm, $E^{259}_{1mg/ml}$ 25.0).

Dilute hydrochloric acid or dilute ammonia causes hydrolysis of thraustomycin yielding an inactive substance (β-thraustomycin).

By gas chromatography and mass spectrometry of the trimethylsilyl (TMS) derivatives of a hydrolysate of thraustomycin (3 N hydrochloric acid) the derivatives of adenine (MW 279)[4] and leucine (MW 275) could be identified. The leucine residue has L-configuration as shown by GC of the TFA-leucine-isopropyl ester on an optically active stationary phase[5]. A third component occurs in two isomers, which can be

Fig. 1. Mass spectrum of the TMS derivative of β-thraustomycin

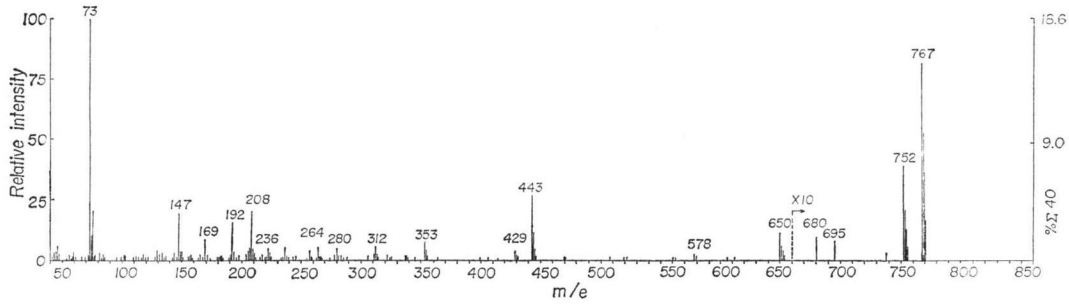
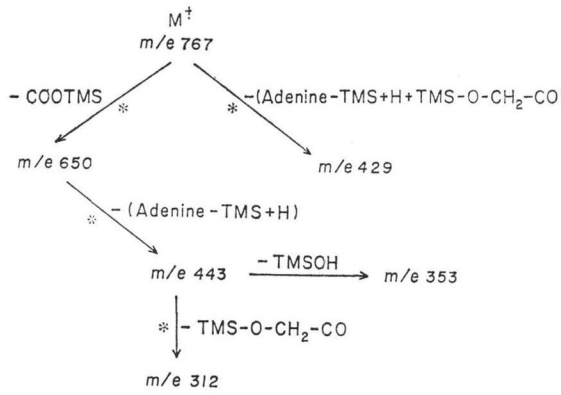


Fig. 2. Fragmentation scheme of the TMS derivative of β-traustomycin



separated on a GC column. Both isomers have identical mass spectra showing a molecular ion of low intensity at mass 650.

The antibiotic itself is not sufficiently volatile for mass spectrometry. However, trimethylsilylation forms a volatile derivative. The complex mass spectrum shows a molecular peak at mass 970. Because of the very low stability of thraustomycin all further investigations were performed with the relatively stable β-thraustomycin. The mass spectrum of the TMS derivative of β-thraustomycin (Fig. 1) shows a molecular ion at *m/e* 767. The mass difference of 203 mass units between the TMS derivatives of thraustomycin and β-thraustomycin implies the loss of a leucine residue with one TMS group. In the hydrolysate of β-thraustomycin adenine and the unknown isomers with molecular weight 650 are present but no leucine can be detected.

For detailed characterization of β-thraustomycin some special derivatives had to be prepared. The reaction with perdeuterated bis-trimethylsilyl-acetamide (d_9BSA)[6] yields a derivative with a molecular peak at *m/e* 812. A mass shift of the molecular ion by 45 mass units *versus* the normal TMS derivative clearly indicates that 5 functional groups are silylated. Deduction of 5 TMS groups leaves a molecular weight of 407 for free β-thraustomycin.

Reaction with a small excess of diazomethane prior to silylation yields a derivative with a molecular weight of 709, This suggests the presence of one carboxylic group in the molecule. The methyl ester group is split off in the mass spectrometer under formation of an intense fragment ion

Table 2. Exact mass values of some fragment ions of the TMS derivative of β-thraustomycin

m/e	Found	Calculated	Error [mm*u*]	Composition
650	650.2617	650.2680	6.3	$C_{27}H_{48}N_5O_6Si_4$
443	443.1704	443.1729	2.5	$C_{19}H_{35}O_6Si_3$
429	429.1566	429.1584	1.8	$C_{18}H_{33}O_6Si_3$
353	353.1239	353.1239	0.0	$C_{16}H_{25}O_5Si_2$
236	236.0962	236.0967	0.5	$C_9H_{16}N_5OSi$
208	208.1028	208.1018	1.0	$C_8H_{14}N_5Si$

Fig. 3. Mass spectrum of the TMS derivative of a fragment of thraustomycin ("sugar component")

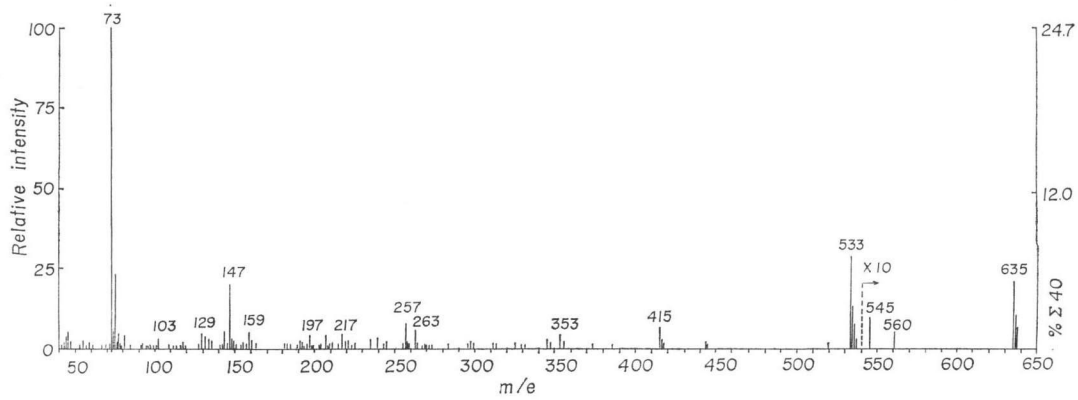


Table 3. Interpretation of some major ions in the mass spectrum of the TMS derivative of the "sugar component" of β-thraustomycin

m/e (TMS)	*m/e* (d_9 TMS)	Interpretation
635	677	M–CH_3
560	596	M–TMSOH
545	578	M–(TMSOH+CH_3)
533	569	M–COOTMS
455	479	M–(2 TMSOH+CH_3)
443	470	M–(TMSOH+COOTMS)
415	442	M–(TMSOH+COOTMS+CO)
353	371	M–(2 TMSOH+COOTMS)
263	272	M–(3 TMSOH+COOTMS)

Table 4. Interpretation of some major ions in the mass spectrum of the main derivative of the "sugar component" of β-thraustomycin after methylation and trimethylsilylationn

m/e	Interpretation
450	M^+
435	M–CH_3
419	M–OCH_3
403	M–(CH_3+CH_3OH)
391	M–$COOCH_3$
387	M–(OCH_3+CH_3OH)
375	M–$CH(OCH_3)_2$
344	M–($CH(OCH_3)_2$+CH_3O)
343	M–($CH(OCH_3)_2$+CH_3OH)
331	M–($COOCH_3$+$HCOOCH_3$)
297	M–(CH_3OH+OCH_3+TMSOH)
160	CH_3>CH_3>CH–$CH(OCH_3)$–CH=C=O
159	$C_7H_{11}O_4$
145	160–CH_3
75	$CH(OCH_3)_2$

at *m/e* 650 (M^+–$COOCH_3$, m* 597).

The same ion is formed in the all-TMS derivative (M^+–COOTMS) and is also confirmed by a metastable transition (m* 551.5). The further fragmentation proceeds from *m/e* 650 to *m/e* 443 by loss of the protonated and mono-silylated adenine residue (m* 303) and from *m/e* 443 to *m/e* 353 by elimination of a trimethylsilanol molecule. From mass 443 also a fragment of 131 mass units is lost, containing one TMS group according to the TMS-d_9 derivative. As shown by high resolution mass spectrometry the fragment 131 has the composition $C_5H_{11}O_2Si$ and a tentative structure of TMS–O–CH_2–C=O. This fragment is also lost together with the protonated adenine moiety directly from the molecular ion

Table 5. Exact mass values of some fragment ions of the "sugar component" after methylation and trimethylsilylation

m/e	Found	Calculated	Error [mmu]	Composition
419	419.1746	419.1736	1.0	$C_{15}H_{31}O_9Si$
391	391.1792	391.1787	0.5	$C_{17}H_{31}O_5Si$
160	160.0720	160.0735	1.5	$C_7H_{12}O_4$

m/e 767 yielding an ion at *m/e* 429 (m* 241). This is a strong indication for a nucleoside with a C-1′ substituted by a CH_2–OH group. The fragmentation is indeed very similar to the mass spectrum of the TMS derivative of 6-amino-9-(β-psicofuranosyl) purine (psicofuranine, angustmycin C)[7,12].

Fig. 2 shows a fragmentation scheme for the TMS derivative of β-thraustomycin. The mass spectrum of TMS-β-thraustomycin shows also some features known from the corresponding adenosine spectrum[8]. The characteristic fragment ions at mass 208 (base +2H) and at mass 236 (base +CH_2O) are of high intensity. Their composition is established by exact mass measurement (Table 2). This observation also suggests that a residue similar to a sugar is connected to adenine.

On the basis of exact mass values the exact molecular weight 407.1077 for β-thraustomycin can be calculated with an elemental composition of $C_{16}H_{17}N_5O_8$.

"Sugar Component"

Mild hydrolysis of β-thraustomycin in 3 N hydrochloric acid (2 hours, 100°C) forms a mixture of adenine and two isomeric products. Their identical mass spectra after trimethylsilylation show a molecular ion at *m/e* 650 (Fig. 3). The interpretation of the most important fragment ions in the high mass range is given in Table 3.

The TMS-d_9 derivative shows a molecular peak at *m/e* 695 and a mass difference of 45 mass units *versus* the normal TMS derivative. From this result a molecular weight of 290 can be calculated for the underivatized "sugar".

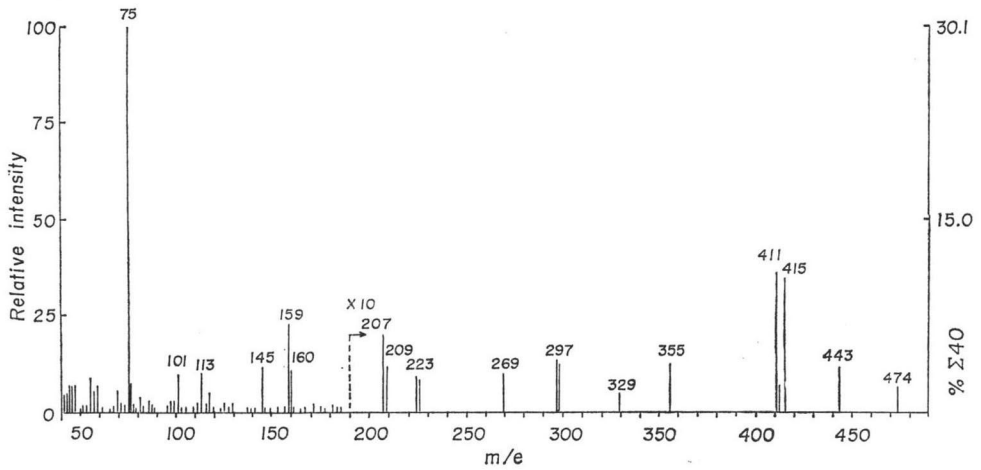
The comparison of various derivatives (TMS, TMS-d_9, (a) diazomethane (b) TMS) gives evidence for one carboxylic acid group and three, possibly four, hydroxy groups to be present in the molecule (Table 3).

Interesting structural information is obtained from the mass spectra of the reaction products of the "sugar component" with a solution of dry hydrogen chloride in methanol (1.25 N) following silylation. In the GC-MS analysis two isomers of molecular weight 476 and a product of molecular weight 450 are found. Again isotope labeling (TMS-d_9, CD_3OD/HCl) is used for determination of the number of reaction sites. The two isomers of molecular weight 476 are formed by methylation of 3 functional groups and trimethylsilylation of 2 additional groups of the "sugar component". The threefold methylation may be explained by reaction of the carboxy group and of two acidic hydroxy groups. It was found that only glycosidic hydroxy groups in sugar molecules are methylated under the conditions applied[9].

In the mass spectrum the methyl ester group is split off giving the intense fragment ion at mass 417. From *m/e* 417 a fragment of 60 mass units is lost ($HCOOCH_3$) to yield an ion at *m/e* 357. This fragmentation is typical for methyl glycosides[9].

The second product formed in this reaction has a molecular ion at *m/e* 450 (Table 4) and is a dimethyl acetal of the "sugar component". By the above mentioned isotope labeling approach it can be shown that 5 methyl groups and only one TMS group are present in the derivative. A large fragment ion at mass 75, which is shifted to *m/e* 81 in the perdeuteromethyl derivative, is indicative for the dimethyl acetal. The loss of 75 mass units from the molecular ion can be observed at *m/e* 375. The presence of a methyl ester group is indicated by an ion at *m/e* 391 (M^+–$COOCH_3$). Intense fragment ions, observed at *m/e* 159 and *m/e* 160, seem to be of structural significance. Isotope labeling makes it evident that both ions contain three methyl groups and no TMS group. The exact mass measurement shows the composition $C_7H_{12}O_4$ for *m/e* 160. A possible structure for this ion is

Fig. 4. Mass spectrum of the "sugar component" of thraustomycin after methylation and trifluoroacetylation



proposed in Table 4.

The structure information obtained from the derivatives after methylation and trimethylsilylation is confirmed by the corresponding trifluoroacetyl (TFA) derivatives. In this case two isomers of molecular weight 428 and a derivative of molecular weight 474 (Fig. 4) are found in the GC/MS investigation. Again the products of MW 428 are formed by methylation of three functional groups and trifluoroacetylation of one hydroxy group. The main product of MS 474 is a dimethylacetal (*m/e* 75) and corresponds to the product of MW 450. The mass difference of twenty-four mass units is due to the difference in molecular weight between a TMS and a TFA group. The interpretation of the most significant ions in the mass spectrum (Fig. 4) is presented in Table 6 and is supported by comparison with the mass spectrum of the perdeuteromethyl derivative. Aside from the dimethylacetal group and the carboxy group the presence of at least one glycosidic hydroxy group is indicated by the loss of fragments of 60 ($HCOOCH_3$) from *m/e* 415 (M^+–$COOCH_3$) to *m/e* 355 and from *m/e* 297 to *m/e* 237. Again, as in the TMS derivative, fragment ions at *m/e* 159 and *m/e* 160 containing three methyl groups are observed. Of several intense fragment ions of derivatives of the "sugar component" exact mass measurements by high resolution mass spectrometry were obtained. From the results, listed in Table 5, deduction of the introduced substituents gives the elemental composition $C_{11}H_{14}O_9$ (MW 290.0638) of the "sugar component". This result is also obtained from exact mass values of the TMS derivative of β-thraustomycin (Table 2) by replacing the adenine by a hydroxy group.

Table 6. Interpretation of some major ions in the mass spectrum of the main derivative of the "sugar component" of β-thraustomycin after methylation and thrifluoroacetylation

m/e (CH_3OH/HCl)	*m/e* (CD_3OD/HCl)	Interpretation
474	—	M
443	455	M–OCH_3
415	427	M–$COOCH_3$
411	420	443–CH_3OH
355	365	M–($COOCH_3$ + $HCOOCH_3$)
329	—	443–TFAOH
298	308	411–TFAO
297	307	411–TFAOH
269	278	297–CO
237	—	297–$HCOOCH_3$
160	169	$C_7H_{12}O_4$
159	168	$C_7H_{11}O_4$
145	—	160–CH_3

Discussion

Of all the investigated antifungal antibiotics many cause inhibition of the growth of mycelium, but only polyoxins[10] show specific action on the formation of zygospores[2]. The stronger action of thraustomycin on zygospore formation is caused by higher sensitivity of (+)-mycelium in comparison to (−)-mycelium, and not by inhibition of any of the important metabolic reactions of zygospore formation. According to ENDO[11] polyoxins specifically inhibit the synthesis of chitin. The reason for the greater inhibition by polyoxins of zygospore formation in comparison to growth of mycelium is unknown.

In the literature until 1972[12] no purine antibiotic was found which is identical with thraustomycin based on its composition of adenine, L-leucine, and the unusual sugar-like compound. From mass spectrometry it is obvious that adenine is substituted in only one site which is most likely the N-9 position and the leucine moiety must be connected to the "sugar component". The structure of the "sugar component" is not clear yet but could be characterized by its elemental composition ($C_{11}H_{14}O_9$) and by identification of one carboxy group, one aldehyde group, one glycosidic hydroxy group and three further hydroxy groups.

Since thraustomycin could so far be isolated in amounts of a few mg only the investigations were restricted to mass spectrometry. We expect to obtain more details of its structure from chemical reactions or by NMR spectroscopy after isolation of larger amounts of the antibiotic.

Acknowledgement

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