48. Studies on the Biosynthesis of Tabtoxin (Wildfire Toxin). Origin of the Carbonyl C-Atom of the β-Lactam Moiety from the C_1-Pool

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Re-isolation of *Pseudomonas tabaci* strain *NCPPB 2730* from its host, the tobacco plant, led to an activation of the bacteria in order to produce the β-lactam dipeptide tabtoxin (Wildfire toxin, **1**). Incorporation of several ^{14}C-labelled amino acids as well as L-[*methyl*-^{13}C]methionine, L-[1,2-$^{13}C_2$]- and L-[3,4-$^{13}C_2$]aspartate, *rac*-[1,2-$^{13}C_2$]glycerol, and [1,2-$^{13}C_2$]acetate into isotabtoxin (**2**) demonstrated that the building blocks of tabtoxin (**1**) are L-threonine, L-aspartate, the Me group of L-methionine and a C_2-unit derived from the C_3-pool *(Fig. 3)*. The Me group of L-methionine provides the carbonyl C-atom of the β-lactam moiety. These findings represent a novel pathway in β-lactam biosynthesis. Mechanistic aspects with respect to the β-lactam ring formation are discussed. A biradical **16** is proposed as an intermediate during the cyclization of a *N*-formyl-α-amino ketone **15**.

Introduction. – Tabtoxin (**1**) is an exotoxin of the phytopathogenic bacterium *Pseudomonas tabaci* and induces the wildfire disease, a leaf-spot disease, on tobacco plants [1]. It is known that *P. tabaci* can lose its ability to produce the phytotoxin, if the bacterium is stored on laboratory media [2]. The determination of the structure of **1**, which consists of L-threonine and the unusual amino acid tabtoxinine-β-lactam, proved to be difficult due to the toxin's instability ($t_{½}$ = 24 h at 25° and pH 7) [3]. The biologically active β-lactam **1** readily undergoes intramolecular transacylation to the stable, but inactive δ-lactam isotabtoxin (**2**).

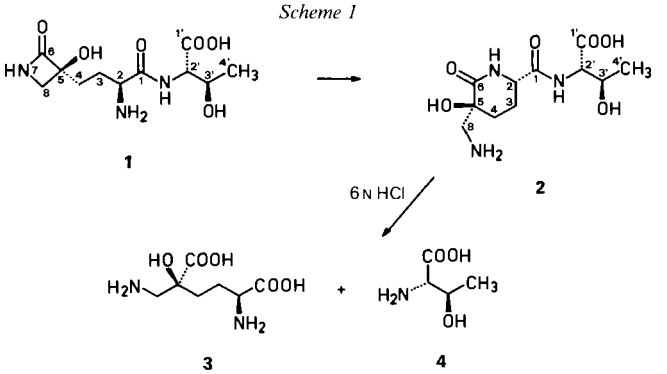
Here, we report on the cultivation of the bacterium and studies on the biosynthesis of **1** using radioactive and ^{13}C-labelled precursors.

Results. – We were initially unable to isolate tabtoxin (**1**) or its isomer isotabtoxin (**2**) from culture broths containing *Woolley*'s medium [1], which had been inoculated with the strain *P. tabaci NCPPB 2730*. Therefore, we decided to re-isolate the bacteria, which were present for a period of 5 days on the tobacco leaf, from its host *(Nicotiana Havanna)* by cutting out a diseased yellow leaf spot and grinding it with a roughened glass rod in *Woolley*'s medium. To re-isolate strains from single colonies, differently diluted samples of the bacterial suspension were plated on an agar minimal medium in petri dishes. After incubation at 28° for 2 days, two morphologically different types of bacterial colonies A and B could be distinguished. Colonies of type A were rather yellow and smaller than those of type B, which were opaque and had a milky appearance. Re-isolates of type-A colonies were able to induce once again the typical chlorotic lesions on tobacco leaves and showed, after their re-isolation from the leaf and plating on agar minimal medium as described above, again both types of colonies, A and B. In contrast, bacteria from type-B colonies caused only a small, necrotic spot on the tobacco plant. The re-isolated bacteria of type B from leaves gave, when plated on agar minimal medium, only colonies of type B.

A control experiment with pieces of healthy leaves excluded the occurrence of infections. After repeated re-isolation of *P. tabaci* from the tobacco plant, we observed a strain development. First, the ratio of the colony types A and B increased in favour of A; secondly, we were able to isolate *ca.* twice the amount of isotabtoxin (**2**) originally present. Hence, an 'activation' of the bacteria seemed to have taken place on its host, the tobacco plant. However, the distinction of the two colony types on agar plates could only be made with *P. tabaci* from strain *NCPPB 2730,* but not with those of strain *ATCC 11528.*

Erlenmeyer flasks containing *Woolley's* medium were inoculated with 'activated' *P. tabaci* and incubated on a rotary shaker at 28°. Radioactive precursors were mixed with the media before inoculation, whereas ^{13}C-labelled compounds were 'pulsefed' in 5 portions every 10–12 h. The cultures were harvested after 3 days yielding 100–200 mg/l of isotabtoxin (**2**) as an artefact of the workup. No effort has been made to isolate **1** in its native form, since the analysis of **2** was representative for the biosynthetic investigations *(Scheme 1).*

Scheme 1



1 **2** **3** **4**

To elucidate potential precursors, we first carried out incorporation experiments with radioactive amino acids. Further information was obtained from the acid-catalysed hydrolysis of the radioactive isotabtoxin (**2**) samples to tabtoxinine (**3**) and threonine (**4**) *(Scheme 1)* showing the relative distribution of radioactivity in both degradation products *(Table 1).*

Table 1. *Incorporation of Radioactive Precursors into Isotabtoxin* (**2**)

Precursors	Absolute incorporation rate [%]	Relative radioactivity in the hydrolysis products [%]	
		Tabtoxinine (**3**)	Threonine (**4**)
L-[U-^{14}C]Lysine	0.53	66	34
L-[U-^{14}C]Lysine[a])	0.47[a])	–[b])	–[b])
L-[U-^{14}C]Threonine	4.2	5	95
L-[*methyl*-^{14}C]Methionine	18	99	1
[2-^{14}C]Glycine	1.2	88	12
L-[U-^{14}C]Glutamic acid	1.7	53	47
L-[U-^{14}C]Aspartic acid	3.2	74	26

[a]) Precursor diluted with inactive material and added at the beginning.
[b]) Not determined.

These experiments indicate that L-threonine, as expected, is the direct precursor of the threonine moiety of the dipeptide. L-[*methyl*-^{14}C]methionine, [2-^{14}C]glycine, and L-[U-^{14}C] aspartic acid are significantly incorporated into the unusual amino acid. However, it cannot be ruled out that the relatively low incorporation rate of [2-^{14}C]glycine is merely due to the metabolic interrelationship between glycine and the C_1-pool. Thus, C(2) of glycine may enter the tetrahydrofolate cycle *via* 5,10-methylene-tetrahydrofolate. On the other hand, it is not surprising that L-[U-^{14}C]aspartic acid is incorporated up to one fourth into the threonine moiety of tabtoxin (**1**), since L-aspartic acid is a known precursor of L-threonine. It seemed likely that L-[U-^{14}C]glutamic acid enters the tricarboxylic-acid cycle, where the radioactivity is distributed statistically. The low absolute incorporation rate of L-[U-^{14}C]lysine suggests that the basic amino acid is either not a specific precursor or is not efficiently taken up by the cells.

Table 2. *Chemical Shifts in the ^{13}C-NMR Spectrum of Isotabtoxin* (**2**) *and Relative Integrals after Incorporation of* L-[methyl-^{13}C]*Methionine*

C-Atom	Chemical shift [ppm][a])	Relative integral	
		Natural abundance	Enriched
C(4')	20.2	1	1
C(3)	22.4	2.3	1.0
C(4)	29.9	1.2	1.2
C(8)	46.6	1.9	1.1
C(2)	55.7	0.9	1.2
C(2')	61.7	2.1	1.0
C(3')	68.5	1.0	1.0
C(5)	69.2	0.9	0.9
C(1)	173.9	0.6	1.1
C(6)	174.3	0.6	31.1
C(1')	177.3	0.5	0.5

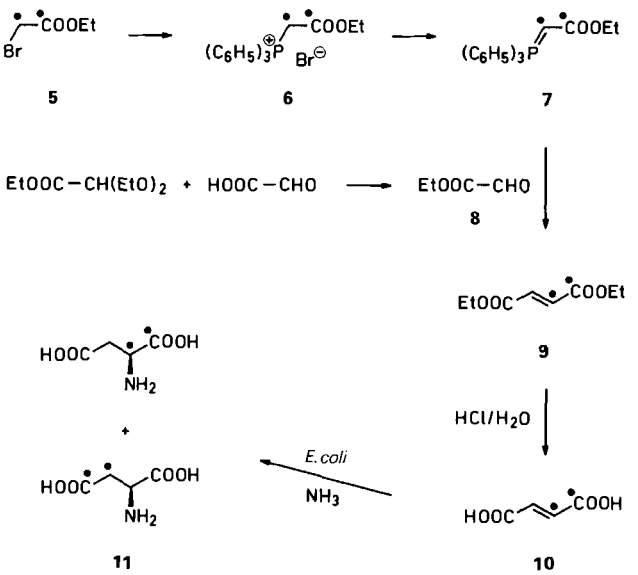
[a]) Assignments have been confirmed by means of ^{1}H,^{1}H-, ^{1}H,^{13}C-, and ^{1}H,^{13}C-long-range correlated spectra.

By the administration of L-[*methyl*-^{13}C]methionine to *P. tabaci*, we confirmed the high incorporation rate found in the radioactive experiment and showed that the β-lactam carbonyl C-atom C(6) was derived from the Me group of methionine *(Table 2)*. The incorporation rate was high enough (63.5%) that, in the proton-noise-decoupled ^{13}C-NMR spectrum, an additional doublet was observed, which is due to the adjacent C(5) with natural-abundance ^{13}C content ($J(^{13}C,^{13}C)$ = 52.3 Hz).

To establish those building blocks of tabtoxin (**1**) which are derived from L-aspartic acid, we prepared a 1:1 mixture of L-[1,2-$^{13}C_2$]- and L-[3,4-$^{13}C_2$]aspartic acid (**11**) *(Scheme 2)*. This labelling pattern of L-aspartic acid allowed us to determine, whether all C-atoms of the amino acid are incorporated. In addition, it offered the possibility to observe ^{13}C,^{13}C-coupling in the isolated labelled sample of the metabolite.

The synthesis started with commercially available ethyl [1,2-$^{13}C_2$]bromoacetate (**5**) which was converted to the stabilized phosphorane **7** *via* the phosphonium salt **6** [4]. A *Wittig* reaction with ethyl glyoxylate (**8**) [5] yielded diethyl [1,2-$^{13}C_2$]fumarate (**9**). Hydrolysis of **9** with 6N HCl led to [1,2-$^{13}C_2$]fumaric acid (**10**). The desired mixture of doubly labelled L-aspartic acids **11** was obtained from [1,2-$^{13}C_2$]fumaric acid (**10**) and NH_3 using

Scheme 2

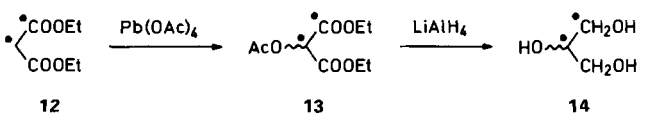


aspartase, which is present in immobilized cells of *E. coli ATCC 11303* [6]. The enantiomeric purity was determined to be 100% by capillary GLC on a chiral stationary phase [7]. The proton-noise-decoupled ^{13}C-NMR spectrum of the enriched isotabtoxin (**2**) sample obtained after incorporation of **11** is shown in *Fig. 1a*. The observed ^{13}C,^{13}C-coupling pattern is consistent with the assumption that two intact units of aspartic acid had been incorporated into tabtoxin (**1**) *(Fig. 2a)*. L-Aspartic acid, as a direct precursor of L-threonine, was incorporated into the threonine moiety of **1**. In addition, L-aspartic acid was established as the biogenetic precursor of the side chain of tabtoxinine-β-lactam.

Because D-glycerate is established as a specific precursor of the C-atoms of the β-lactam ring of clavulanic acid [8], and the biogenetic origin of the two remaining C-atoms in the β-lactam ring of **1** is still unknown, we synthesized racemic [1,2-$^{13}C_2$]-glycerol *(Scheme 3)*. Diethyl [1,2-$^{13}C_2$]malonate (**12**) was oxidized with $Pb(OAc)_4$ to yield racemic diethyl 2-acetoxy[1,2-$^{13}C_2$]malonate (**13**). Reduction of **13** with $LiAlH_4$ gave racemic [1,2-$^{13}C_2$]glycerol (**14**) [9].

The labelled compound **14** was administered to growing cultures of *P. tabaci*. In the proton-noise-decoupled ^{13}C-NMR spectrum of isotabtoxin (**2**) *(Fig. 1b)*, we observed that, except for the carbonyl C-atom of the β-lactam ring already known to be derived from the Me group of methionine, every C-atom showed signals due coupling with the adjacent C-atoms derived from the same precursor molecule. The coupling pattern in the

Scheme 3



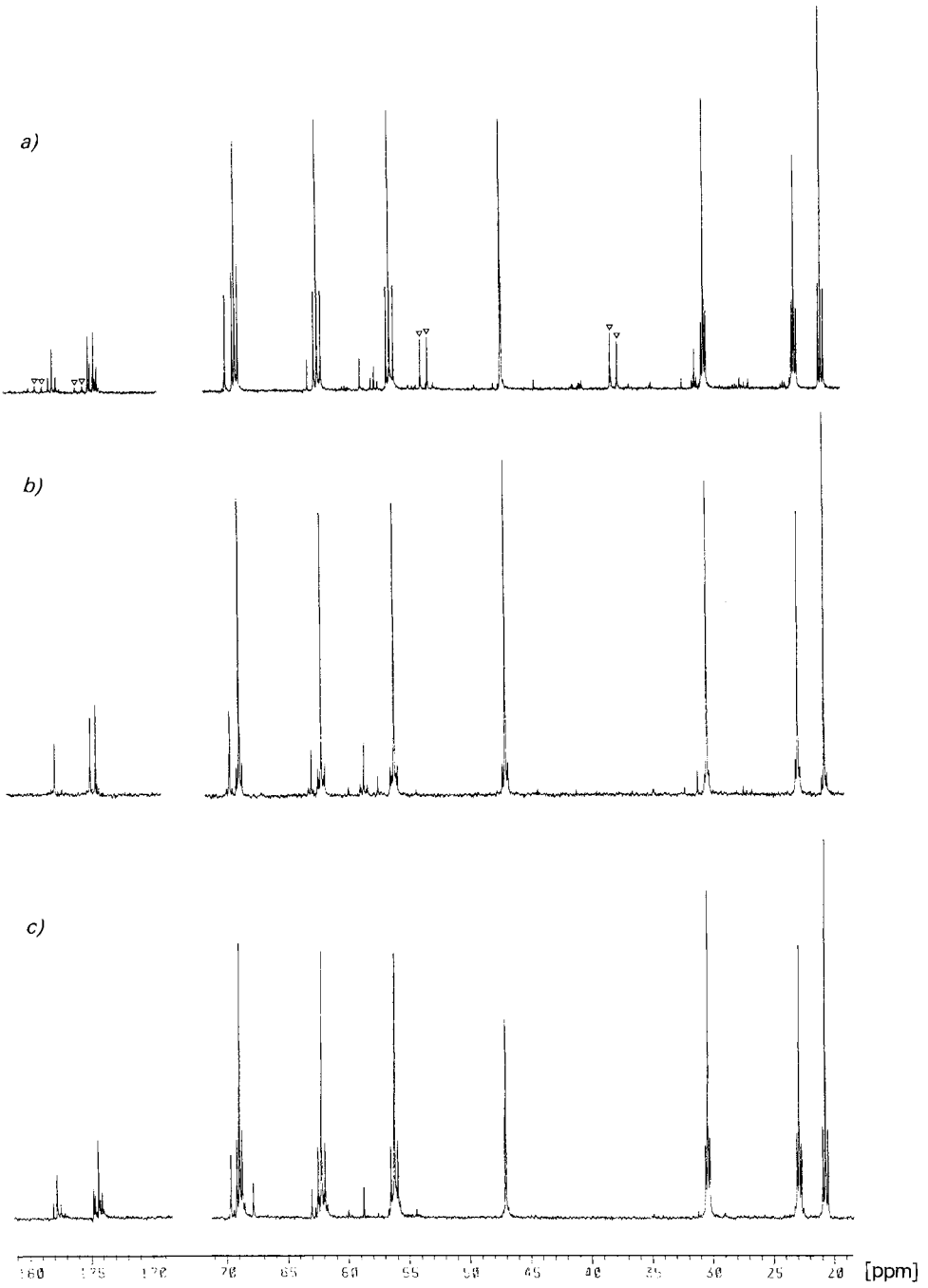


Fig. 1. *Proton-noise-decoupled ^{13}C-NMR spectra of enriched isotabtoxin* (**2**) *after incorporation of* a) L-*[1,2-$^{13}C_2$]- and* L-*[3,4-$^{13}C_2$]aspartic acid* (**11**; ▽ = signals from precursor), b) *racemic [1,2-$^{13}C_2$]glycerol, and* c) *[1,2-$^{13}C_2$]-acetate*

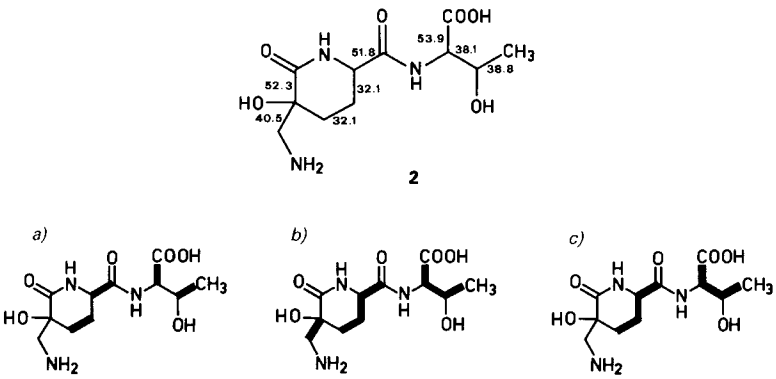


Fig. 2. *$^{13}C,^{13}C$-Coupling constants in isotabtoxin* (**2**) *and coupling pattern in enriched specimens of* **2** *after incorporation of* a) L-*[1,2-$^{13}C_2$]- and* L-*[3,4-$^{13}C_2$]aspartic acid* (**11**), b) *racemic [1,2-$^{13}C_2$]glycerol, and* c) *[1,2-$^{13}C_2$]acetate*

enriched sample of **2** *(Fig. 2b)* revealed two C_4-units and an additional C_2-unit for the two β-lactam C-atoms. The fact that three neighboring C_2-units form a C_4-unit can readily be explained in view of the fact that racemic [1,2-$^{13}C_2$]glycerol (**14**) may enter the tricarboxylic acid cycle in two different ways. One of these routes leads, with [2,3-$^{13}C_2$]pyruvate as an intermediate, to doubly labelled acetate. The latter is subsequently converted into aspartate and threonine. Since all coupling signals in **2** are similarly small, glycerol does not appear to be the specific precursor of the β-lactam C-atoms. However, the metabolisms of the direct precursor of the considered C_2-unit and of glycerol must be connected.

As the acetate is a product of the glycolysis and, thus, a potential precursor of the C_2-unit of the β-lactam ring, an incorporation experiment with [1,2-$^{13}C_2$]acetate seemed to be reasonable. However, no coupling between the two corresponding β-lactam C-atoms, C(5) and C(8), was observed in the proton-noise-decoupled ^{13}C-NMR spectrum of the labelled specimen of **2** *(Fig. 1c)*. In addition, no enhancement of the corresponding NMR signals could be detected after standardization. Of course, the 8 C-atoms of the side chain and part of the δ-lactam ring showed the expected coupling pattern *(Fig. 2c)*. Acetate has entered the tricarboxylic-acid cycle and has labelled aspartic acid as well as threonine.

The results obtained by our incorporation experiments involve the entire C-skeleton of tabtoxin (**1**) and are summarized in *Fig. 3*.

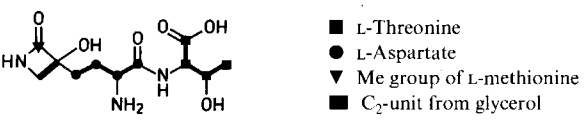


Fig. 3. *Building blocks in the biosynthesis of tabtoxin* (**1**)

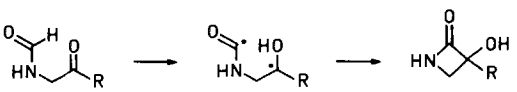
Discussion. – After *P. tabaci* had been stored on laboratory media and had lost its ability to produce tabtoxin (**1**), bacteria of strain *NCPPB 2730* could be activated on their host, the tobacco plant, in order to produce again **1**. Even a strain development was observed. According to *Gasson*, genes essential for toxin production are lost, and this loss may be the result of spontaneous curing of plasmid DNA [10]. Our observations support

the hypothesis [10], that plasmid DNA is involved in tabtoxin production and formation of chlorosis on the tobacco leaf. Additionally, exchange of genetic information appears to occur between host and parasite. The observed strain development may be due to an enhanced replication of plasmids, while *P. tabaci* is growing on its host, or may alternatively be explained by gene amplification.

In the course of several incorporation experiments with radioactive amino acids, L-threonine was established as a direct precursor of the threonine moiety of tabtoxin (**1**). In comparison to other secondary metabolites derived from amino acids, the observed absolute incorporation rate of L-[U-^{14}C]threonine (4.2%) is rather low, even if a possible enhancement through strain development is considered. For example, L-[U-^{14}C]-phenylalanine is incorporated into cytochalasin B and D with rates of 10.4% and 14.2% [11]. Nevertheless, an incorporation rate of 4.2% must be considered as highly significant [12]. Therefore, and because the biosynthesis of threonine is well known, the corresponding moiety of **1** might serve as an internal standard for the interpretation of the incorporation of labelled aspartate, glycerol, and acetate into the tabtoxinine moiety. Thus, L-aspartic acid is established as the biogenetic origin of the side chain of tabtoxinine-β-lactam.

Furthermore, the Me group of methionine was found to provide the carbonyl C-atom of the β-lactam ring. This evidence is rather surprising, since a similar interrelationship between the C_1-pool and the β-lactam ring does not seem to exist in the biosynthesis of other β-lactam metabolites. Hence, the question is raised, as to how β-lactam ring formation might proceed. An explanation may be the cyclization of a *N*-formyl derivative formed by direct formylation of an open-chain amine or, alternatively, by methylation followed by oxidation to a formyl group. An interesting synthetic equivalent to such a cyclization has been found by *Wehrli* in 1980 [13][1]). Thus, UV irradiation of *N*-phenacylformamide (**15**, R = Ph) leads to the corresponding β-lactam **17** (R = Ph), the formyl H-atom being transferred to the O-atom of the carbonyl group *(Scheme 4)*. A hypothetical mechanism for the β-lactam ring closure in the biosynthesis of tabtoxin (**1**) may be formulated according to the mechanism of a similar photoprocess [14], a biradical of type **16** being the intermediate *(Scheme 4)*. A plausible intermediate in a reaction sequence of this kind may be N^{ε}-formyl-5-oxo-L-lysine (**15**, R = $CH_2CH_2CHNH_2COOH$) or a derivative thereof (*e.g.*, N^{ε}-formyl-5-oxo-L-lysyl-L-threonine). However, in view of the current state of our investigations, any statement concerning the moment at which β-lactam cyclization in tabtoxin biosynthesis might occur has to remain speculative.

Scheme 4



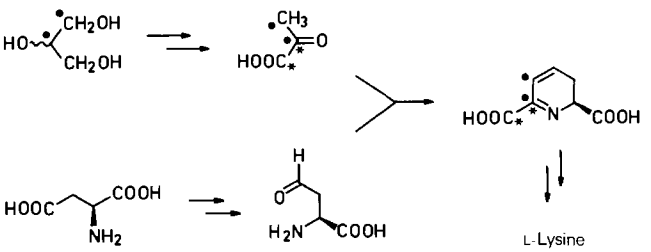
15 **16** **17**

Concerning the remaining two ring-C-atoms, incorporation of [1,2-$^{13}C_2$]glycerol (**14**) showed that these must be derived from a C_2-unit which is metabolized *via* the C_3-pool. Since enzymes normally differentiate between enantiotopic groups, and since racemic **14** has been applied, it is very likely that the direct precursor of the considered C_2-unit is

[1]) We thank Prof. *A. Eschenmoser*, ETH-Zürich, for drawing our attention to this publication.

derived from one of the two labelled enantiomers only. At present, it is only possible to rule out acetate as an intermediate, since [1,2-$^{13}C_2$]acetate is obviously not incorporated into the β-lactam ring of tabtoxin (**1**), not even after degradation *via* the tricarboxylic acid cycle. On the other hand, it is still conceivable, although L-lysine is inefficiently incorporated, that the biosynthesis of the tabtoxinine moiety of **1** proceeds in part along the lysine pathway. This assumption would explain the intact incorporation of L-aspartic acid as well as the C_2-unit originating from racemic [1,2-$^{13}C_2$]glycerol but not from acetate. Thus, glycerol may be metabolized to pyruvate which enters lysine biosynthesis by condensation with aspartate semialdehyde *(Scheme 5)*. It must then be assumed that tabtoxinine biosynthesis branches off from some intermediate of the lysine pathway. Further investigations in order to test this hypothesis are currently being carried out in our laboratory.

Scheme 5



In summary, we can not conclude yet, if tabtoxin (**1**) is only derived from amino acids which form a peptide precursor, as it is the case in the biosynthesis of penicillin [15], and nocardicin A [16], or if there exists a mixed biogenetic origin as found in clavulanic acid [8] and in thienamycin [17]. In the case of clavulanic acid, D-glycerate is established to be a direct precursor of the four-membered ring. Furthermore, acetate is involved in the β-lactam ring formation of thienamycin. On the basis of these findings, a common pathway for the biogenesis of the β-lactam moieties in natural products can be ruled out. Our results support this conclusion, since the incorporation of the Me group of methionine into the β-lactam carbonyl C-atom reveals a new aspect in the biosynthesis of β-lactams.

The support of these investigations by the *Swiss National Science Foundation* is gratefully acknowledged. We thank Prof. Dr. *F. Meins, Friedrich Miescher Institute, Ciba-Geigy AG*, Basel, for the gift of tobacco plants. We are indebted to *Spectrospin AG*, Fällanden, for running the two-dimensional NMR spectra.

Experimental Part

1. *General.* ^{14}C- and ^{13}C-labelled compounds were purchased from *Medipro AG*, Teufen; *Amersham International plc*, England; *Aldrich Chemical Company*, USA; *New England Nuclear*, USA. The origin of *P. tabaci* strain *NCPPB 2730* was the *National Collection of Plant Pathogenic Bacteria*, England; that of *P. tabaci* strain *ATCC 11528* and *E. coli* strain *ATCC 11303* the *American Type Culture Collection*, USA. Stock cultures of *P. tabaci* were maintained on agar slants containing *NBY* medium [10] and stored at 4°. Agar slants of *E. coli* (2.3% nutrient agar *(Difco)*, 0.5% NaCl) were kept at the same conditions. The tobacco plants *(Nicotiana Havanna)* was placed at our disposal by Prof. *F. Meins (Friedrich-Miescher Institute, Ciba-Geigy*, Basel). The org. extracts were dried over Na_2SO_4 and evaporated under reduced pressure below 50°. Column chromatography: silica gel *60* (63–200 μm,

Merck); cellulose microcristallin *Avicel pH-101* (50 μm, *Fluka*); *Dowex 50W-X8* (300–850 μm, *Fluka*); solvent proportions in *v/v*. TLC: silica gel *60 F_{254} (Merck)*; detection with UV, I_2, $KMnO_4$ or ninhydrin soln. HPLC: 5 μm (*μBondapak C_{18}*, 3.9 × 300 mm, *Waters*) using 0.1% CF_3COOH in H_2O. M.p.: *Kofler* Block; corrected. NMR: *Varian-EM-360* spectrometer (^1H, 60 MHz), *Bruker-WH-90* spectrometer with *Fourier* transform (^1H, 90 MHz; ^{13}C, 22.63 MHz), *Bruker-WH-360* (^1H, 360 MHz; ^{13}C, 90 MHz). IR: *Perkin Elmer* model *177* grating spectrometer. MS: *VG-70-250* instrument. Radioactivity: *Radio TLC scanner* model *RTLC (Labotron), Nuclear Chicago* scintillation counter.

2. *Activation of* Pseudomonas tabaci. Of a 2-day-old preculture of *P. tabaci NCPPB 2730* (see *Exper. 3*), 20–40 μl were pipetted onto a pricked tobacco leaf *(Nicotiana Havanna)*. After 5 days, a diseased leaf spot was cut out and ground in *Woolley's* medium [1] with a roughened glass rod. The resulting bacterial suspension was diluted (1:10, 1:100, 1:1000) with 1% saline. Samples (0.1 ml) of differently diluted solns. were plated on *PMS*-agar medium [10] in petri dishes for purification. After incubation at 28° for 2 days in the dark, bacterial colonies of types A and B appeared. Those of type A were used to inoculate agar slants containing *NBY* medium [10]. The latter were incubated for 4 days at 28° in the dark and used for inoculation of precultures.

3. *Production of Tabtoxin* (**1**) *and Isolation of Isotabtoxin* (**2**). Precultures contained 50 ml of *Woolley's* medium [1] in 100-ml *Erlenmeyer* flasks and were shaken, after inoculation with activated bacteria from agar slants, for 16–24 h at 28° on a rotary shaker (200 rpm) in the dark. Then, 10 ml of a preculture were used to inoculate 250-ml portions of *Woolley's* medium contained in 500-ml *Erlenmeyer* flasks. Incubation followed as described above for 3 days. Aq. solns. of radioactive precursors (pH was adjusted if necessary) were aseptically added to the cultures before inoculation, sterile solns. of ^{13}C-labelled compounds (pH adjusted) were administered to the growing cultures in 5 portions every 12 h. The culture medium (1 l) was freed of cells by centrifugation (10000 *g*, 40 min), filtrated (*Whatman GF/F*, 0.7 μm), concentrated to 50 ml at 50° *in vacuo* and freeze-dried. The residue was extracted with MeOH (200 ml) overnight at 4°, and the supernatant was evaporated to dryness *in vacuo* in the presence of *Celite*/cellulose microcristallin 1:1 (0.5 g each). The powder was loaded onto a column (3 × 39 cm) of the same *Celite*/cellulose mixture, which had previously been prepared in pentane and equilibrated with PrOH/H_2O 5:1. The column was operated with 150 ml of PrOH/H_2O 4:1 followed by PrOH/H_2O 3:1 making use of a MPLC apparatus (*model B-680, Büchi AG*, Switzerland). Crude **2** was eluted with a retention volume of 650–1100 ml (TLC on silica gel; PrOH/H_2O 3:1; R_f 0.15). The appropriate fractions were combined, evaporated, dissolved in 15 ml of H_2O, and treated with charcoal (1 g/0.5 g). After stirring for 10 min, the charcoal was filtered off, and the filtrate evaporated in the presence of silica gel (40–63 μm; 1.5 g/0.5 g). The powder was transferred to a flash column (silica gel) previously equilibrated with PrOH/H_2O 5:1. The column was eluted with 50 ml of PrOH/H_2O 4:1 followed by PrOH/H_2O 3:1 yielding **2** (100–200 mg/l culture) as an oil. Radioactive samples of **2** were further purified applying HPLC (5 μm *μBondapak C_{18}*; 0.1% of CF_3COOH in H_2O, 1 ml/min; UV detection (225 nm); t_r 4.2 min). IR (KBr): 3400–3250, 1665, 1595. ^1H-NMR (360 MHz, D_2O): 1.2 (*d*, *J* = 6, CH_3(4')); 2.0 (*m*, CH_2(4)); 2.1–2.5 (*m*, CH_2(3)); 3.2, 3.4 (*A* and *B* of *AB*, *J* = 13.2, CH_2(8)); 4.2 (*d*, *J* = 6, H–C(2')); 4.3 (*m*, H–C(3')); 4.4 (*m*, H–C(2)). ^{13}C-NMR (90 MHz, D_2O): *cf. Table 2*. FAB^+-MS: 290 (MH^+). FAB^--MS: 288 ($[M-H]^-$).

4. *Hydrolysis of* **2** *to Tabtoxinine* (**3**) *and Threonine* (**4**). For 1 h, 48 mg (0.17 mmol) of **2** was refluxed in 6 ml of distilled 6N HCl (110°). The mixture was separated on prep. TLC (silica gel) with EtOH/H_2O 4:1 (R_f (**3**) 0.3, R_f (**4**) 0.04). Zones of **3** and **4** were scraped off and suspended in 0.3N NH_3. Filtration and evaporation yielded **3** and **4**. ^1H-NMR of **3** (90 MHz, D_2O): 1.9–2.5 (*m*, CH_2CH_2); 3.2, 3.4 (*A* and *B* of *AB*, *J* = 13.2, CH_2NH_2); 3.9 (*m*, $CHNH_2$). ^1H-NMR of **4** (90 MHz, D_2O): 1.3 (*d*, *J* = 6, CH_3); 3.5 (*d*, *J* = 6, H–C(2)); 4.2 (*m*, H–C(3)).

The mixtures obtained after hydrolysis of the radioactive isotabtoxins were chromatographed on TLC. The radioactivity of **3** and **4** was measured on these plates with a *Radio-TLC-Scanner* or alternatively by liquid scintillation counting of samples of **3** and **4** which had been scraped off the TLC plate and suspended in scintillation cocktail/H_2O 9:1.

5. *([carbonyl-^{13}C]Ethoxycarbonyl[^{13}C]methyl)triphenylphosphonium Bromide* (**6**). As previously described [4], 1 g (6 mmol) of ethyl [1,2-$^{13}C_2$]bromoacetate (**5**; *Aldrich*) was converted to 2.42 g (94%) of **6**. M.p. 147–148°. ^1H-NMR (60 MHz, $CDCl_3$): 1.0 (*t*, *J* = 7, CH_3); 4.0 (*q*, *J* = 7, CH_3CH_2O); 5.5 (*d*, $^2J(^1H,^{31}P)$ = 14, PCH_2CO); 7.4–8.2 (*m*, $(C_6H_5)_3$).

6. *([carbonyl-^{13}C]Ethoxycarbonyl[^{13}C]methyliden)triphenylphosphorane* (**7**). The conversion of 2.42 g (5.6 mmol) of **6** to 1.75 g (90%) of **7** was carried out as previously described [14] with the following modifications. The suspension obtained after precipitation of **7** was filtered, the residue dissolved in CH_2Cl_2, and the soln. evaporated and dried *in vacuo* (0.01 Torr, 7 h). M.p. 117–119°. ^1H-NMR (60 MHz, $CDCl_3$): 1.0 (*t*, *J* = 7, CH_3); 2.9 (*s*, P = CH); 4.0 (*q*, *J* = 7, CH_3CH_2O); 7.2–7.9 (*m*, $(C_6H_5)_3$).

7. *Ethyl Glyoxylate* (**8**). From an exchange reaction between 13 g (0.14 mol) of glyoxylic acid monohydrate and 26 ml (0.14 mol) of ethyl diethoxyacetate followed by reaction with 18 g of P_2O_5, **8** was prepared according to [5]. The mixture was stored at 4° under Ar and distilled to obtain **8** as required. B.p. 29–30°/17 Torr. ^{1}H-NMR (60 MHz, $CDCl_3$): 1.3 (*t*, *J* = 6, CH_3); 4.3 (*q*, *J* = 6, CH_2O); 9.3 (*s*, CHO).

8. *Diethyl [1,2-$^{13}C_2$]Fumarate* (**9**). Under Ar, 1.75 g (5.0 mmol) of **7** in 2 ml of dry CH_2Cl_2 were added to 1 g (9.8 mmol) of **8** in 10 ml of dry CH_2Cl_2. The mixture was stirred for 90 min, concentrated *in vacuo*, and passed through a short column of silica gel with petroleum ether/Et_2O 1:1 to give 845 mg (98%) of a mixture, which was shown to contain 93% of **9** and 7% of diethyl maleate by GLC. The latter substance was isomerized to the (*E*)-compound under the acidic conditions of the next step. ^{1}H-NMR (60 MHz, $CDCl_3$): 1.3 (*t*, *J* = 8, 2 CH_3); 4.2 (*q*, *J* = 8, 2 CH_2O); 6.9 (*s*, CH=CH).

9. *[1,2-$^{13}C_2$]Fumaric Acid* (**10**). Overnight, 845 mg (4.9 mmol) of **9** were refluxed in 1 ml of H_2O and 2 ml of 2N HCl. The resulting suspension of **10** was cooled, evaporated, and freed of HCl by repeated evaporation from H_2O. After drying *in vacuo* (0.01 Torr, P_2O_5, 12 h), 563 mg (98%) of **10** were obtained. M.p. 280° (subl.). ^{1}H-NMR (60 MHz, DMSO): 6.6 (*s*, CH=CH); 11.8 (br. *s*, 2 COOH).

10. *1:1 Mixture of* L-*[1,2-$^{13}C_2$]- and* L-*[3,4-$^{13}C_2$]Aspartic Acid* (**11**). *Immobilization of* E. coli ATCC 11303. Inocula were prepared from Agar slants of *E. coli* (2.3% nutrient agar *(Difco)*, 0.5% NaCl). Precultures were grown in 100-ml *Erlenmeyer* flasks each containing 50 ml of the following medium (g/l): ammonium fumarate 30 (22.8 g of fumaric acid in 200 ml of H_2O, pH adjusted to 8.6 with 25% NH_4OH soln.); K_2HPO_4 2; $MgSO_4 \cdot 7\,H_2O$ 0.5; $CaCO_3$ 0.5; corn steep liquor *(Sigma)* 40. After an incubation period of 16–24 h at 37° on a rotary shaker (200 rpm) in the dark, 10-ml portions of a preculture were used to inoculate 250-ml portions of the same medium contained in 500-ml *Erlenmeyer* flasks. Incubation was carried out at the same conditions for 24 h. The culture medium was centrifuged (2500 *g*, 10 min), the cells (7 g wet weight/10.5 l culture) were washed 3 times with 1% saline, suspended in 7 ml of H_2O (50% bacterial suspension) and maintained at 45°. One volume of 50% bacterial suspension was added to 2 volumes of 5% *carrageenan (Sigma C-1263)* in H_2O at 45° with stirring. After mixing and cooling, the immobilized bacterial gel-matrix was ground, hardened in 2% KCl overnight, and washed with H_2O. Centrifugation (2500 *g*, 10 min) gave 40 g of wet gel.

Enzyme Reaction. First, 563 mg (4.8 mmol) of **10** were dissolved in a soln. of 3 mg of $MgCl_2$ in 1.2 ml of 25% NH_4OH soln. and 3.3 ml of H_2O. Then, the mixture (pH 8.6) was shaken with 3 g of gel at 50° in the dark for 24 h (200 rpm). The gel-matrix was then removed by centrifugation (2500 *g*, 10 min) and washed twice with H_2O. The combined solns. were evaporated at 50°, and the residue was dissolved in 2N HCl and concentrated again. Purification was achieved by chromatography on a 3 × 12-cm column of *Dowex 50W-X8* previously equilibrated with aq. formic acid of pH 1.5. The crude hydrochloride was dissolved in a minimal amount of the same solvent and applied to the column. The column was washed with 500 ml of H_2O and eluted with 1M pyridinium formate pH 5.0. Ninhydrin-positive fractions (TLC: silica gel; EtOH/H_2O 4:1; R_f 0.55) were pooled, evaporated, taken up in H_2O, and evaporated again yielding 512 mg (80%) of **11** (0.01 Torr, P_2O_5, 96 h). The optical purity was determined to be 100% e.e. by GLC of the diisopropyl *N*-(pentafluoropropionyl)aspartate [7] on a chiral stationary phase (*WCOT* fused silica *Chirasil-L-Val*, 25 m, 0.22 mm i.d., film thickness 0.12 μm, *Baker*). Sample purity was determined similarly by internal standardization using DL-aspartic acid. ^{1}H-NMR (60 MHz, D_2O, NaOD): 2.6 (*AB* of *ABX*, *m*, CH_2); 3.7 (*X* of *ABX*, *m*, CH). ^{13}C-NMR (22.63 MHz, D_2O, NaOD, proton-noise decoupled): 36.9 (*d*, *J* = 51, C(3)); 52.5 (*d*, *J* = 54, C(2)); 174.5 (*d*, *J* = 54, C(1)); 177.8 (*d*, *J* = 51, C(4)). MS: 136 (MH$^+$), 92 (MH$^+ - CO_2$), 91 (MH$^+ - {}^{13}CO_2$), 90 ($M^+ - CO_2$), 89 ($M^+ - {}^{13}CO_2$).

11. *Racemic Diethyl 2-Acetoxy-[1,2-$^{13}C_2$]malonate* (**13**) [9]. To 0.5 g (3.1 mmol) of diethyl [1,2-^{13}C$_2$]malonate (**12**) in 10 ml of benzene were added 2.2 g (4.9 mmol) of $Pb(OAc)_4$ with stirring under Ar. The mixture was refluxed for 3¼ h and then washed with cold 1N NaOH (1 × 10 ml) and H_2O (3 × 10 ml). The combined org. layers were dried and evaporated giving 595 mg (88%) of **12**. ^{1}H-NMR (60 MHz, $CDCl_3$): 1.3 (*t*, *J* = 7, 2 CH_3); 2.2 (*s*, $COCH_3$); 4.2 (*q*, *J* = 7, 2 CH_2); 5.4 (*s*, H–C(2)).

12. *Racemic [1,2-$^{13}C_2$]Glycerol* (**14**). Reduction of 595 mg (2.7 mmol) of **13** with 0.38 g (10 mmol) of $LiAlH_4$ in abs. Et_2O was carried out according to [9] with some modifications. The excess of $LiAlH_4$ was decomposed with 2 ml of cold 2N HCl followed by 3 ml of cold conc. HCl instead of H_2O. After continuous extraction with Et_2O in a *Kutscher-Steudel* apparatus for 2 weeks, 216 mg (87%) of **14** were obtained. ^{1}H-NMR (60 MHz, D_2O): 3.6 (*m*, 5 H). ^{13}C-NMR (22.63 MHz, D_2O, proton-noise decoupled): 63.4 (*d*, *J* = 41, $^{13}CH_2OH-{}^{13}CHOH$; *s*, $^{13}CH_2OH-CHOH$); 72.9 (*d*, *J* = 41, $^{13}CH_2OH-{}^{13}CHOH$; *s*, $CH_2OH-{}^{13}CHOH$). CI-MS: 112 (MNH$_4^+$, base peak).

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