STRUCTURE OF THURINGIENSIN, THE THERMOSTABLE EXOTOXIN FROM *Bacillus thuringiensis*

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Degradation of thuringiensin and isolation of structural components is reported. Some derivatives of the "disaccharidic" component of thuringiensin were subjected to a detailed ^{1}H-NMR analysis. Attachments of the particular structural components of thuringiensin were determined with the use of spectral methods. The present degradation along with the subsequent work of other authors show that our original proposal of the structure *Ia* for thuringiensin was correct.

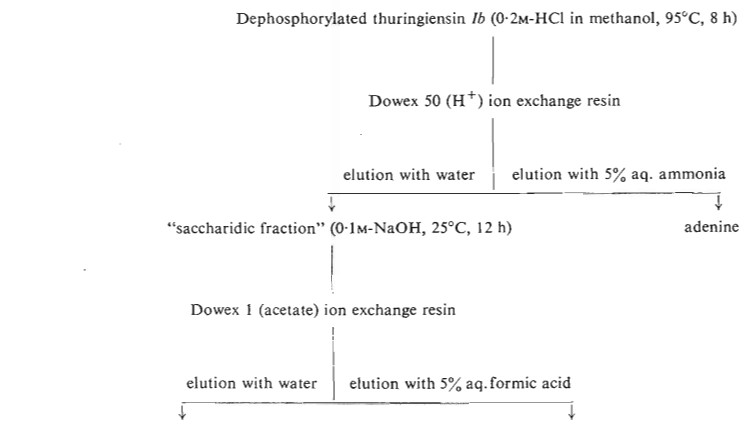
The thermostable exotoxin produced by *Bacillus thuringiensis*[1] and named thuringiensin* is a specific inhibitor of the DNA-dependent RNA polymerase, one of the key enzymes in the transmission of genetic information. Thuringiensin acts[2,3] as competitive inhibitor of the ATP incorporation into RNA. This interesting mechanism of thuringiensin activity prompted us to elucidation of its structure.

In a preliminary communication[5], thuringiensin was assigned the structure *Ia* on the basis of a stepwise degradation. A similar degradation was effected in the same year by Bond and coworkers[6,7] but was not accompanied with any proposal of the structure. Our structural proposal is also presented in a review on the thermostable exotoxin published by Bond and Boyce[1]. On the basis of the synthesis of some degradation products, the structure of thuringiensin was rigorously established[8–11] in this Institute and confirmed by total synthesis[12–15]. In addition to the above mentioned papers[8–11], the original structural proposal[5] (formula *Ia*) has been recently supported by a spectral analysis of thuringiensin performed by French investigators[16] and extended in some points as follows: *a*) The α-configuration of the glucosidic bond was established[9,11]; *b*) The *R*-configuration on the $C_{(2)}$ carbon atom of allaric acid bound by a glucosidic bond was determined[10,11]; *c*) By means of ^{13}C-NMR spectrum, the phosphate bond position in the allaric moiety of thuringiensin was unambiguously determined[16]. In the present

* Kim and Huang[4] proposed the designation thuringiensin A for the free acid *Ia* and the designation thuringiensin B for the lactone of this acid. It would be much more advantageous to our opinion to reserve the name thuringiensin for the free acid *Ia*.

paper*, the chemical degradation of thuringiensin is described in detail and the structure of thus-obtained fragments is determined.

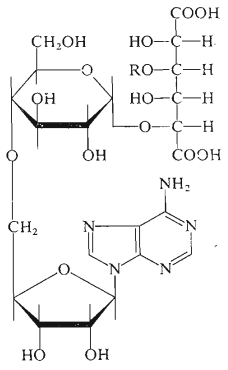
The dephosphorylated thuringiensin *Ib* (*cf.*[17]), obtained by the action of alkaline phosphatase on thuringiensin (*Ia*) was subjected to the acid-catalysed methanolysis and the resulting fragments isolated according to Scheme 1. Adenine was separated on Dowex 50 (H^+) ion exchange resin and the remaining mixture of neutral substances was subjected to alkaline hydrolysis in order to hydrolyse the esters present. After the alkaline hydrolysis, the mixture was chromatographed on Dowex 1 (acetate) ion exchange resin and separated into the neutral fraction F_1 and the acidic fraction F_2. On the basis of preliminary results, fraction F_1 appeared to be a mixture of the four possible anomeric glycosides *II*. Since the chromatographic separation of this original mixture failed, the anomeric glycosides *II* were converted to a mixture of the isopropylidene derivatives *III* which were then chromatographed on silica gel to afford three chromatographically homogeneous fractions. The mass spectra of these fractions were analogous and exhibited a peak (M − 15 at mass 365) characte-



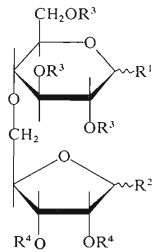
SCHEME 1

* The extended version of the above mentioned preliminary communication[5] on the structure of thuringiensin was presented as a plenary lecture on the VIIth International Symposium on Natural Substances 1969, Riga, Soviet Union (*cf.*[18]).

ristic of acetonides. As the main component of the mixture of the isopropylidene derivatives *III*, the acetonide *IV* was used in determination of the structure of the saccharidic moiety of thuringiensin.



Ia; R = $\overset{\overset{O}{\|}}{P}(OH)_2$
Ib; R = H



II; $R^1 = R^2 = OCH_3$, $R^3 = R^4 = H$
III; $R^1 = R^2 = OCH_3$, $R^3 = H$, $2R^4 = C(CH_3)_2$
IV; $R^1 = \alpha\text{-}OCH_3$, $R^2 = \beta\text{-}OCH_3$, $R^3 = H$, $2R^4 = C(CH_3)_2$
V; $R^1 = \alpha\text{-}OCH_3$, $R^2 = \beta\text{-}OCH_3$, $R^3 = Ac$, $2R^4 = C(CH_3)_2$
VI; $R^1 = OCH_3$, $R^2 = OH$, $R^3 = R^4 = H$
VII; $R^1 = R^2 = OH$, $R^3 = R^4 = H$

The acetonide *IV* was transformed into the tri-O-acetyl derivative *V*, the structure of which was established by means of the ^{1}H-NMR spectrum, see Fig. 1. In the lower part of this figure, the ^{1}H-NMR spectrum of compound *V* is shown (in deuteriochloroform at 100 MHz). In the high field region, typical signals of methyl group protons are present, namely, two singlets of the O-isopropylidene group, three singlets of acetyl groups, and two singlets of methoxy groups. From the presence of these methyl group signals the structure of a di-O-methyl diglycoside triacetate monoacetonide was inferred. The remaining portion of the spectrum is formed by signals of the O—CH type protons. As indicated by a detailed analysis by means of frequency swept decoupling experiments, this portion of spectrum consists of superposition of two isolated spin systems, namely, one six-spin system and one seven-spin system. On the basis of continuity of chemical shifts and coupling constants, the symmetry of these spin systems may be for example expressed as $A_2BMM'X$ and

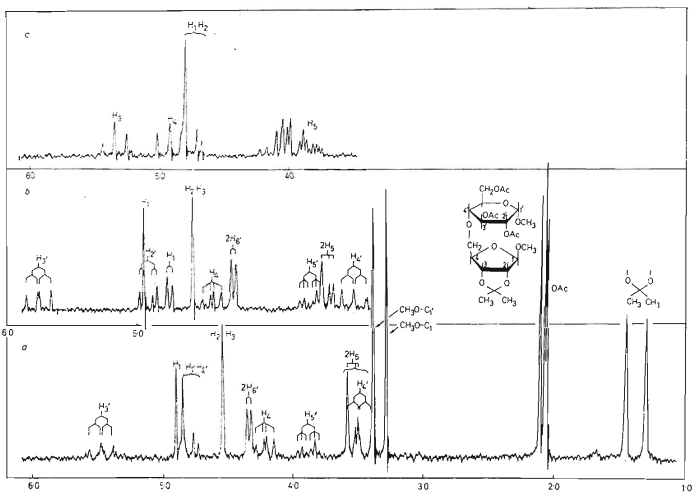


FIG. 1

100 MHz-^{1}H-NMR Spectrum of the Isopropylidene Derivative *V*

a Measured in deuteriochloroform (tetramethylsilane as internal standard); *b* the sugar part of the bottom spectrum in hexadeuteriobenzene (100 MHz); *c* the sugar part of the methyl 2,3,4,6-tetra-O-acetyl-α-D-glucopyranoside spectrum in deuteriochloroform (100 MHz).

A_2LMRYZ and it may be inferred (also with respect to the approximate values of coupling constants) that one component of the "diglycoside *V*" is a pentofuranoside and the other one a hexopyranoside.

The six-spin system of the pentafuranoside portion of the spectrum consists of a singlet of the H_1 proton, a two-proton singlet of H_2 and H_3 protons, and a quartet of the H_4 proton as a part of the A_2B system, the A_2 part of which consists of the H_5 protons. Signals of the H_5 protons coincide with signals of one proton from the other spin system. The whole spin system can be seen in a spectrum taken in hexadeuteriobenzene (Fig. 1). The characteristic features of this spin system are as follows: *a*) The vicinal interaction values $J_{1,2} = 0$ Hz and $J_{3,4} \neq 0 \ll 1$ Hz suggest the *trans* configuration of H_1—H_2 and H_3—H_4 protons; *b*) the considerable resistance of "internal" chemical shifts towards the solvent effect of benzene suggest a low polarity of the O—CH bonds in the furanoside portion of the molecule; the bonds are more likely of the ethereal type.

It might be thus concluded that the O-isopropylidene group is attached to the furanoside at positions $C_{(2)}$ and $C_{(3)}$, *i.e.*, that the configuration of H_2—H_3 protons is *cis* and that the furanoside component is methyl 2,3-O-isopropylideneribofuranoside. This assumption is in a good accordance with the known spectra of 2,3-O-isopropylideneribofuranosides and may be thus considered as very probable. The ribofuranoside component would be attached to the hexapyranoside through the $C_{(5)}$ carbon atom by an ethereal bond as also suggested by the chemical shift of $H_{(5)}$ protons.

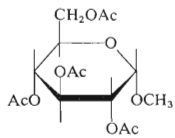
The seven-spin A_2LMRYZ system of the hexopyranoside portion of the spectrum consists of the low field RYZ part formed by $H_{2'}$, $H_{1'}$, and $H_{3'}$ protons. The other high field A_2LM part contains a doublet of two $H_{6'}$ protons (A_2), a doublet of triplets due to the $H_{5'}$ proton, and a quartet of the $H_{4'}$ (LM) proton. The RYZ and A_2LM parts are connected by a single vicinal interaction of $H_{3'}$ and $H_{4'}$ protons. Signals of the $H_{4'}$ proton coincide with those of the H_5 proton and the whole spin system is completely separated in the spectrum taken in hexadeuteriobenzene. The characteristic features of this spin system are as follows: *a*) High values of coupling constants $J_{2',3'}$, $J_{3',4'}$, and $J_{4',5'}$ (9—10 Hz), indicating for the corresponding protons a diaxial configuration which is typical of the $^1C_4$ configuration of glycopyranosides. *b*) The $H_{1'}$ and $H_{2'}$ protons afford a degenerate five-line subspectrum of the YZ part of the RYZ subspectrum while the $H_{3'}$ proton (R) exhibits a basic triplet with a series of combination lines. In the spectrum taken in hexadeuteriobenzene, the "internal" chemical shift of $H_{1'}$ and $H_{2'}$ protons increases and the degeneration disappears. The spectrum of the RYZ subsystem may be observed in the normal form as a $H_{1'}$ doublet, $H_{2'}$ doublet of doublets, and $H_{3'}$ quartet. Such a degeneration is typical of the ^{1}H-NMR spectrum of methyl 2,3,4,6-tetra-O-acetyl-α-D-glucopyranoside (*VIII*) taken for the sake of comparison in deuteriochloroform (Fig. 1). As inferred from comparison of the ^{1}H-NMR spectra of compounds *V* and *VIII*, the

hexapyranoside spectral portion of the "diglycoside *V*" is in good accord with the ^{1}H-NMR spectrum of compound *VIII* ($J_{1',2'}$ = 3·6 Hz) and corresponds to an axial-equatorial $H_{2'}$—$H_{1'}$ interaction. The only difference consists in symmetry of the spin system of $H_{6'}$ and $H_{5'}$ protons and especially in the position of the $H_{4'}$ proton quartet. The proton on the $C_{(4)}$ carbon atom occurs at 5·03 ppm with the glycoside *VIII* and at 3·46 ppm with the "diglycoside *V*". This shift indicates that the bond on the $C_{(4')}$ carbon atom of the "diglycoside *V*" is of an ethereal type. From all these observations on the ^{1}H-NMR spectrum of the acetyl derivative *V* it may be concluded that the "disaccharide" moiety of thuringiensin consists of ribose and glucose linked together anomalously by means of an ethereal bond extending from the $C_{(5)}$ carbon atom of ribose to the $C_{(4')}$-carbon atom of glucose.

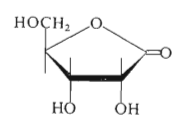
The structure of the "diglycosidic" component of thuringiensin was also confirmed by a partial hydrolysis of the anomeric mixture of methyl glycosides *II* with the formation of the anomeric mixture *VI* which was in turn transformed either to a mixture of anomeric 1-phenylflavazole[19] derivatives *IX* or to a mixture of anomeric ribonolactones *XII*. For purposes of ^{1}H-NMR spectra, the anomeric mixtures *IX* and *XII* were transformed into the acetyl derivatives *X* and *XIII*. The advantage of this approach consisted in disappearence of one of the two anomeric centres. The original problem of a mixture containing four anomers was reduced to the content of two anomers: such a mixture can be directly analysed by ^{1}H-NMR spectra. Thus, as inferred from decoupling experiments, the mixture *X* consisted mainly of the β-anomer *XI* while in the mixture *XIII*, the α-anomer *XIV* predominated. The formation of 1-phenylflavazole derivatives by reaction of *o*-phenylenediamine and phenylhydrazine with a pentofuranose or hexofuranose proceeds under elimination of all hydrogen atoms on $C_{(1)}$, $C_{(2)}$, and $C_{(3)}$ carbon atoms of the saccharide skeleton, *i.e.* from standpoint of ^{1}H-NMR spectra, the original skeletal six-spin furanose system is reduced to a R—CH(OR′)—CH_2OR″ three-spin system and the seven-spin hexopyranose system shrinks to a four-spin R—CH(OR′)—CH(OR″)—CH_2OR‴ system.

As indicated by ^{1}H-NMR spectra of model 1-phenylflavazole derivatives of the ribose *XV* and the glucose *XVI*, the proton H_1 signal from the original $C_{(4)}$ carbon atom of the saccharide exhibits in acetylated 1-phenylflavazole derivatives a relatively high downfield shift (6·81 ppm with compound *XV* and 6·77 ppm with compound *XVI*). This circumstance is advantageous in the use of 1-phenylflavazole derivatives for investigations on structures of saccharides bound by non-glycosidic bonds with the aid of ^{1}H-NMR spectra making easier assignment of the H_1 proton and thus identification of the whole proton system of the linear saccharidic component by means of decoupling experiments. In the ^{1}H-NMR spectrum of compound *X* in deuteriochloroform, the H_1 proton forms a quartet at 6·59 ppm (J_1 = 4·5 Hz and J_2 = 7·0 Hz) as the X part of the isolated ABX system with the AB part of H_2 protons at 4·47 ppm (dd, J_1 = 7 Hz, J_2 = 10 Hz) and at 4·27 ppm (dd, J_1 = 4·5 Hz,

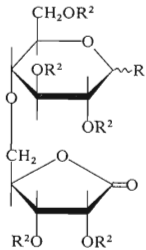
J_2 = 10 Hz). The presence of this ABX system confirms in compound *VI* the existence of a pentofuranose attached by a non-glycosidic ethereal bond to the $C_{(5)}$ carbon atom. The lower value of the H_1 chemical shift in compound *X* when compared with that in compound *XV* is due to the absence of the β-acyl shift; in addition to the lower value of the average shift of the CH_2 group in compound *X* (4·37 ppm) than



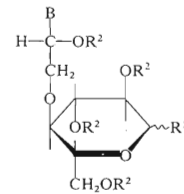
VIII



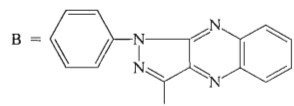
XVII

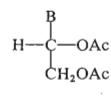


XII; $R^1 = OCH_3$, $R^2 = H$
XIII; $R^1 = OCH_3$, $R^2 = Ac$
XIV; $R^1 = \alpha\text{-}OCH_3$ $R^2 = Ac$

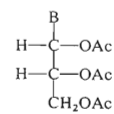


IX: $R^1 = OCH_3$, $R^2 = H$
X: $R^1 = OCH_3$, $R^2 = Ac$
XI: $R^1 = \beta\text{-}OCH_3$, $R^2 = Ac$





XV



XVI

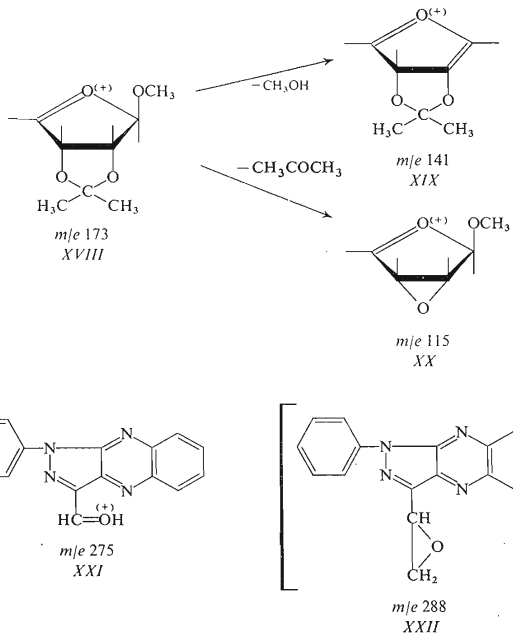
in compound *XV* (4·56 ppm), the presence of an ethereal oxygen on the $C_{(2)}$ carbon atom is directly indicated by a lowered value of the geminal interaction $J_{2a,2b} = 10$ Hz in compound *X* (referred to $^2J = 12{\cdot}5$ Hz in methane; *cf.* $^2J_{2a,2b} = 12{\cdot}5$ Hz in compound *XV*). The other portion of the ^{1}H-NMR spectrum of compound *X* is consistent with the assumed presence of a glucopyranoside attached to the $C_{(4')}$ carbon atom by an ethereal bond ($H_{4'}$ at 3·71 ppm), the $^1C_4$ conformation, and the equatorial OCH_3 group on $C_{(1')}$, *cf.* the major signal of OCH_3 at 3·46 ppm (>80%) and the minor signal at 3·36 ppm.

In the case of compound *XII*, the IR spectrum indicated the presence of a γ-lactone (1 769 cm^{-1}) in accordance with the assumed presence of a pentofuranose in compound *VI*. A detailed analysis of the ^{1}H-NMR spectrum of compound *XIII* by means of decoupling experiments confirmed the occurrence of an isolated five-spin $ABMX_2$ system with a characteristic continuity of vicinal interactions ($J_{AB} = J_{2,3} = 6{\cdot}5$ Hz; $J_{AM} = J_{1,3} = 0$ Hz; $J_{BM} = J_{3,4} = 0{\cdot}6$ Hz) similar to γ-ribonolactone (*XVII*), ($J_{2,3} = 5{\cdot}3$ Hz, $J_{3,4} = 0{\cdot}8$ Hz). The remaining portion of the ^{1}H-NMR spectrum of compound *XIII* is consistent with the assumed presence of a glucopyranoside attached to $C_{(4')}$ through an ethereal bond ($H_{4'}$ at 3·48 ppm), the $^1C_4$ conformation, and the axial OCH_3 group on $C_{(1')}$ (major signal of OCH_3 at 3·39 ppm, 80%, minor signal at 3·48 ppm) similarly to compound *V*.

Steric relations of the ribose residue (as a component of the sugar moiety of thuringiensin) were inferred from the resemblance between ORD spectra of lactones *XII* and *XVII*. On the basis of a dependence[20] between ORD spectra of simple sugar lactones and their configuration on the $C_{(2)}$ carbon atom it may be assumed that the pentose component of the lactone *XII* and thus of thuringiensin itself is derived from D-ribose. In order to determine steric relations of the glucose portion of the "disaccharide" moiety of thuringiensin, the dephosphorylated thuringiensin *Ib* was subjected to hydrolysis in 50% aqueous trifluoroacetic acid at 95°C. The resulting reducing "disaccharide" was tentatively ascribed the structure *VII* since glycosidation with 0·1M-HCl in methanol afforded a mixture of anomeric glycosides *II*. The sugar *VII* proved to be considerably resistant towards acidic hydrolysis since 16 hours of heating at 95°C in 50% aqueous trifluoroacetic acid were required to obtain a low yield of a monosaccharide which was identified as glucose by paper chromatography and gas chromatography of the corresponding trimethylsilyl derivative. As established by a positive enzymatic assay[21], the thus-obtained glucose belongs to the D-series.

The mass spectra of the acetonide *IV* and 1-phenylflavazole derivative *IX* are also in accordance with the above inferred structure of the "disaccharide" portion of the thuringiensin (*Ia*) molecule. The mass spectrum of the acetonide *IV* contained a peak of the maximum mass at *m/e* 365 corresponding to the M − 15 ion formed by elimination of the methyl group from the acetonide system. The presence of the glycoside and acetonide groupings in the ribose component of compound *IV* is reflected

in a relative abundance of the ion *XVIII* of mass 173. Although the corresponding metastable ions have not been found, the ion *XVIII* presumably undergoes a further fragmentation with the loss of a methanol or acetone molecule and with the formation of ions *XIX* and *XX* of mass 141 and 115. The 1-phenylflavazole derivatives of both the monosaccharides and oligosaccharides are suitable for mass-spectral measurements since the molecular peaks are well discernible[22-24]. The mass spectrum of the 1-phenylflavazole derivative *IX* exhibits a molecular peak at mass 482 corresponding to the "disaccharidic" character of the sugar moiety of thuringiensin. The spectrum also exhibits peaks at masses 275 and 228, ascribable to structures *XXI* and *XXII*. The structure of the "disaccharidic" component of thuringiensin was also confirmed by identity of ^{1}H-NMR spectra of compound *V* obtained both by degradation of thuringiensin and by an unambiguous synthesis[8].



Fraction F_2 (Scheme 1) was identified as allaric acid on the basis of IR spectrum which is identical with that of authentic allaric acid and markedly different from that of galactaric acid. These two acids were taken into consideration as potential structural components of thuringiensin since the ^{1}H-NMR spectrum of the acidic component F_2 exhibited only two broad singlets corresponding to symmetrical hexaric acids (at 4·17 ppm and 3·95 ppm in hexadeuteriodimethyl sulfoxide). Our observation of allaric acid as the structural component of thuringiensin was later on confirmed by Bond and coworkers[7,8] and French authors[16].

The UV spectrum of both the parent thuringiensin (*Ia*) and its dephosphorylated derivative *Ib* indicates that adenine is substituted at position 9 (the character of UV spectra of adenine derivatives substituted at positions 1, 3 or 7 is quite different from that of 9-substituted derivatives[25]). The presence of a 9-substituted adenine moiety in thuringiensin (*Ia*) was inferred by Bond[7] from differences of chemical shifts due to H_2 and H_8 protons on the purine ring system[26].

The attachment of adenine to the anomeric centre of D-ribose in the molecule of thuringiensin (*Ia*) is suggested by the ^{1}H-NMR spectrum of compound *Ib* (Fig. 2) in hexadeuteriodimethyl sulfoxide after the addition of trideuterioacetic acid (at 100 MHz). In the downfield part of the spectrum at 8·44 and 8·30 ppm two broadened singlets of two adenine ring system protons are present. The proton of the $C_{(1')}$ nucleoside centre is attributable to the doublet at 5·98 ppm ($J_{1',2'}$ = 5 Hz) while the vicinal proton corresponds to the triplet at 4·56 ppm ($J_{2',1'} = J_{2',3'}$ = 5 Hz, confirmed by decoupling experiments). These parameters of $H_{1'}$ and $H_{2'}$ protons are in good accordance with those of the $H_{1'}$ proton (5·91 ppm, $J_{1',2'}$ = 6·30 Hz) and the $H_{2'}$ proton (4·62 ppm, $J_{2',1'}$ = 6·30 Hz, $J_{2',3'}$ = 4·9 Hz) in the ^{1}H-NMR spectrum of adenosine which was measured under analogous conditions for purposes of comparison; it may be therefore assumed that the configuration of the nucleoside bond in thuringiensin (*Ia*) is identical with that in adenosine. Concerning the lower $J_{1',2'}$ value of compound *Ib* (5 Hz) when compared with adenosine (6·30 Hz), such a decrease is obviously typical (*cf.* the comparative investigations[27,28] on ^{1}H-NMR spectra of adenosine derivatives) of the $C_{(5')}$—OH→$C_{(5')}$—OR substitution (R = = alkyl, acyl) also occurring in thuringiensin. Moreover, the existence of a ribose--adenine bond in thuringiensin is in agreement with measurements on the hydrolysis rate of the nucleoside bond of thuringiensin in 0·1M-HCl at 80°C. This hydrolysis rate is similar to that of the nucleoside bond in adenosine[29] and adenylic acid and is by orders of magnitude higher than the hydrolysis rate of the nucleoside bond in 9-β-D--glucopyranosyladenine. The β-configuration at the anomeric centre of the D-ribose moiety in thuringiensin may also be inferred from resemblance of the ORD spectra of thuringiensin and ATP.

Owing to the relatively ready removal of allaric acid by methanolysis of the dephosphorylated thuringiensin *Ib* it may be assumed that allaric acid is attached by

a glycoside bond to the anomeric centre of D-glucose. The attachment of allaric acid to D-glucose by an ester bond may be excluded since two free carboxylic groups are present in compound *Ib* according to the alkalimetric titration. The glycoside bond between D-glucose and allaric acid was assigned the α-configuration by Bond and coworkers[7] on the basis of the ^{1}H-NMR spectrum of compound *Ib*. The doublet at 5·17 ppm ($J_{1'',2''}$ = 3 Hz) was identified[7] as a signal of the anomeric proton in the glycoside residue. This assignment is somewhat problematic but, nevertheless, appears correct. This doublet is visible on Fig. 2 at 4·86 ppm (J = 3·5 Hz). The corresponding vicinal proton lies in the region of about 3·30 ppm as established by decoupling experiments. It may be noted for purposes of comparison that the H_1 signal in the hexadeuteriodimethyl sulfoxide solution of methyl α-D-glucopyranoside lies at 4·52 ppm (J = 3 Hz) and of methyl β-D-glucopyranoside at 4·55 ppm (J = = 7 Hz; *cf.*[30]); with methyl 4,6-benzylidene-α-D-glucopyranoside[31], the signal is located at 4·99 ppm (J = 3·8 Hz). An unequivocal evidence for the α-configuration at the anomeric centre of D-glucose in the molecule of thuringiensin (*Ia*) has been recently presented in this Institute[9–11]. On the basis of an observation that a pair

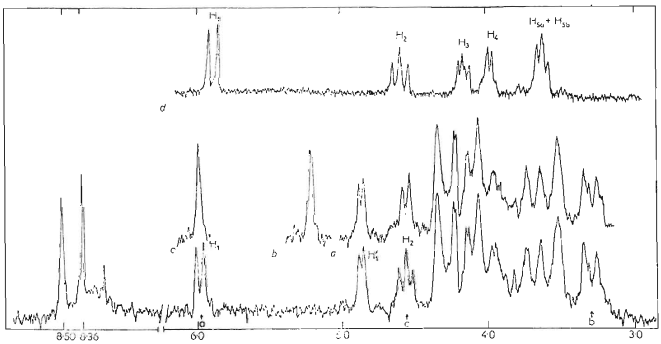
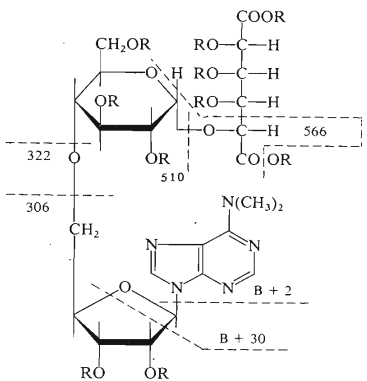


FIG. 2

100 MHz-^{1}H-NMR Spectrum of the Dephosphorylated Thuringiensin *Ib*

Solvent, hexadeuteriodimethyl sulfoxide with the addition of trideuterioacetic acid; hexamethyldisiloxane (HMDS) as internal standard; scale related to tetramethylsilane using δ_{HMDS} equal to 0·06 ppm. Additional traces: *a* bottom trace after irradiation of the position *a*; *b* H_1, signal after irradiation of the position *b*; *c* H_1 signal after irradiation of the position *c*; *d* the sugar part of adenosine ^{1}H-NMR spectrum measured under analogous conditions for purposes of comparison.

of lactones is obtained from compound *Ib* in acidic media, the glucose residue was believed[5] to be attached to one of the two hydroxylic functions adjacent to the carboxylic group of allaric acid. This assumption has been recently confirmed by the use of two independent procedures[9–11] demonstrating also the *R*-configuration of the allaric acid carbon atom bearing the glucose residue.



XXIII; R = CH_3

The molecular weight was estimated[17] on the basis of absorbance at 259 nm, namely, 725 with thuringiensin (*Ia*) and 652 with compound *Ib*. Values in the range of 825–850 (*Ia*) and 725–750 (*Ib*) were given by Bond and coworkers[7]. An attempt was made to determine the exact molecular weight value of thuringiensin (*Ia*) by means of mass spectroscopy. For this purpose, the dephosphorylated thuringiensin *Ib* was converted by the action of methyl iodide and sodium hydride in dimethylformamide[32,33] into the permethyl derivative *XXIII* which was isolated from the reaction mixture by a combination of the ion exchange chromatography and absorption chromatography in 5% yield. The low yield of the methylation process is probably due to elimination reactions proceeding in the allaric portion of the molecule of compound *Ib* under conditions of the methylation. As shown by Posternak[34], methylation of allaric acid is accompanied by elimination of β-hydroxylic groups with the formation of geometrical isomers of α,α'-dimethoxymuconic acid. From the similar UV spectra of the permethyl derivative *XXIII* and 6-dimethylamino-9-β-D-ribofuranosylpurine[35,36], the permethyl derivative *XXIII* was inferred to contain similarly a dimethylamino group at position 6 of the purine ring system. This con-

clusion is supported by the observation that methylation of adenosine with methyl iodide and sodium hydride in dimethylformamide affords 6-dimethylamino-9-(2,3,5--tri-O-methyl-β-D-ribofuranosyl)purine[11]. When the experimental work was accomplished, a report of McCloskey[37] was published on the preparation of adenosine permethyl derivative by the same procedure (for the methylation of the adenine ring in the modified molecule of dephosphorylated thuringiensin see also ref.[11]). It is worth of mention that the methylation of adenosine in aqueous media or in dimethylacetamide exhibits a different course; the primary product is represented by 1-methyladenosine which undergoes rearrangement in alkaline media with the formation of 6-methylamino-9-β-D-ribofuranosylpurine; further methylation affords 1,N^6-dimethylaminoadenosine or its O-methyl derivative[38,39]. A similar course of methylation was observed with 2'-deoxyadenosine[40]. The UV spectrum of 1,N^6-dimethyladenosine[38] and its pH dependence considerably differ from the UV data of isomeric 6-dimethylaminopurine derivatives[35].

The mass spectrum of the permethyl derivative *XXIII* exhibits a molecular peak at *m/e* 789 corresponding to the calculated value of molecular weight for the structure *XXIII*. Another weak but distinct peak (*m/e* 803) corresponding to the molecular weight of a homologue may also be observed in the mass spectrum of compound *XXIII*. It remains to determine whether this homologue results by C-methylation of the dephosphorylated thuringiensin *Ib* or by methylation of a thuringiensin homologue which could be present in a small amount in the naturally occurring material. The observed molecular weight value of the permethyl derivative *XXIII* is capable of correlation with that obtained[11] in mass spectral measurements of the permethylated analogue of the dephosphorylated thuringiensin containing an allitol residue instead of the allaric acid. As it may be inferred from the above mass spectra, the dephosphorylated thuringiensin *Ib* exhibits the molecular weight 621 and the phosphate *Ia* shows the value 701. The latter value of thuringiensin (*Ia*) is in accordance with that recently determined by French authors[16] by means of mass spectroscopy with the use of the field desorption method. The fragmentation pattern of the permethyl derivative *XXIII* is in accord with the structure ascribed to compound *Ib* on the basis of chemical degradations.

The position of the phosphate residue in the molecule of thuringiensin (*Ia*) was determined indirectly on the basis of periodic acid oxidation. Thus, 2 mol of periodic acid are taken up after 6 h by thuringiensin (*Ia*) and 4·2 mol of periodic acid are consumed by the dephosphorylated compound *Ib*. As indicated by this difference in periodic acid uptake, the phosphate residue present in thuringiensin (*Ia*) interferes with two *cis*-diol systems and should be thus most probably situated at position 4‴. This conclusion has been recently supported by French authors[16] in measurements of the ^{13}C-NMR spectrum of thuringiensin from *B. thuringiensis rhodesia*.

EXPERIMENTAL

Materials and Methods

Thuringiensin (*Ia*) was prepared by fermentation of *B. thuringiensis* var. *gelechiae* AUCT. and isolated by the reported procedure[17]. UV spectrum (in 0·01M-HCl): λ_{max} 260 nm (log ε 4·085). Optical rotation: $[\alpha]_D^{25}$ +30·9° (*c* 0·5; water). ^{1}H-NMR spectrum: see Fig. 3. ORD spectrum (in 0·01M-HCl): peak $[\Phi]_{236}$ +1940°, trough $[\Phi]_{263}$ −560°.

Dephosphorylated thuringiensin *Ib* was prepared from thuringiensin (*Ia*) by alkaline phosphatase and the product isolated on Dowex 1 (formate) ion exchange resin[17]. UV spectrum (in 0·01M-HCl): λ_{max} 260 nm (log ε 4·130). ^{1}H-NMR spectrum: see Fig. 2. For the electrophoretical behaviour of the dephosphorylated thuringiensin *Ib* and the parent thuringiensin *Ia* see Table I.

The ^{1}H-NMR spectra were measured on a Varian HA 100 apparatus at 100 MHz (chemical shifts δ are expressed in ppm). The mass spectra were taken on MCH 1303 (Leningrad, Soviet Union), AEI-MS 902 and AEI-MS 9 spectrometers. The IR spectra were recorded on a UR 10 (Carl Zeiss, Jena) apparatus, the UV spectra on a CF 4 Optica Milano apparatus, and the ORD spectra on a Jasco Model ORD/UV-5 spectropolarimeter. Gas chromatography was performed on an Argone Pye apparatus with a SE-30 (10%) column at 200°C.

Paper chromatography was carried out on Whatman 1 paper in the solvent systems S_1, ethyl acetate–1-butanol–acetic acid–water (7 : 3 : 3 : 1), and S_2, 1-butanol–pyridine–water (5 : 3 : 2). Thin-layer chromatography was performed on ready-for-use Silufol UV_{254} (Kavalier Glassworks, Votice, Czechoslovakia) silica gel sheets and Kieselgel G (Merck, Darmstadt) silica-gel-coated glass plates in the solvent systems S_3, ethyl acetate–acetic acid–water (10 : 3 : 2); S_4, ethyl acetate–pyridine–water (8 : 2 : 1); and S_5, ethyl acetate–acetone (5 : 3). Electrophoresis was carried out on paper Whatman 1 at 25 V cm^{-1} for 90 min in the buffer solutions E_1, 0·05M triethylammonium borate (pH 6·8); E_2, pyridine–acetic acid–water (4 : 13 : 983) (pH 4·2); and E_3, 0·05M sodium

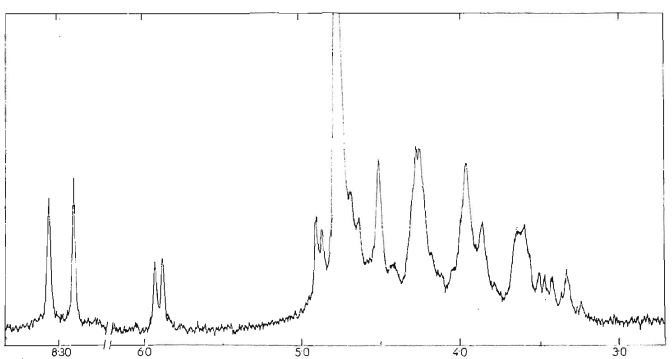


FIG. 3

100 MHz-^{1}H-NMR Spectrum of Thuringiensin (*Ia*)

Thuringiensin (22 mg) in 1M-LiOD/D_2O (0·3 ml); tert-butyl alcohol as internal standard; scale related to tetramethylsilane using δ tert-butyl equal to 1·22 ppm.

hydrogen citrate (pH 3·8). The UV-absorbing substances were detected under the Chromatolite lamp. Reducing saccharides were detected[41] with 2,3,5-triphenyltetrazolium salt solution or silver nitrate in aqueous ammonia; glycosides were detected with a solution of aniline and diphenylamine in dilute phosphoric acid[42]. Substances containing the *cis*-diol system were detected with a solution of sodium periodate and benzidine[43]. Spots on silica-gel-coated glass plates were also detected with sulfuric acid. Phosphates were detected according to ref.[44,45]

Methanolysis of Dephosphorylated Thuringiensin *Ib*

A mixture of dephosphorylated thuringiensin *Ib* (16·7 mg) and 0·2M-HCl in methanol (10 ml) was heated at 95°C for 8 h in a sealed tube. The mixture was then diluted with methanol (10 ml), neutralised with Dowex 1 (acetate) ion exchange resin, filtered, and the filtrate concentrated under diminished pressure to the volume of about 5 ml. The concentrate was applied to a column (1 × 5 cm) of Dowex 50 (H^+) ion exchange resin and the column eluted with water (50 ml) to obtain the "saccharidic fraction". The subsequent elution with 5% aqueous ammonia and evaporation of the eluate yielded 1·9 mg of a residue which was identified as adenine by means of UV and IR (KBr) spectra. The "saccharidic fraction" was evaporated, the residue dissolved in 0·05M-NaOH (10 ml), the solution kept at room temperature for 12 h, neutralised with Dowex 50 (H^+) ion exchange resin, and the resin filtered off. The filtrate was adjusted to pH 9·0 with dilute aqueous ammonia and applied to a column (1 × 5 cm of Dowex 1 (acetate) ion exchange resin. Elution of the column with water (50 ml) and evaporation of the eluate yielded 12·1 mg of the neutral fraction F_1. For the chromatographic and electrophoretic behaviour of this fraction see Table II. Periodic acid oxidation of fraction F_1 (pH 6·8 at 25°C); moles of periodic acid (time in min) given: 1·00 (5), 1·06 (180), 1·27 (360), 1·78 (1440). The subsequent elution of the above column with 5% aqueous formic acid (50 ml) and evaporation of the eluate yielded 3·1 mg of the crystalline fraction F_2. Recrystallisation from a little water yielded a substance, m.p. 195—198°C, undepressed on admixture with authentic allaric acid[46]. ^{1}H-NMR spectrum (hexadeuteriodimethyl sulfoxide): 4·17 and 3·95 (broadened singlets of CH groups). IR spectrum (KBr) of the substance from fraction F_2 is identical with that of allaric acid and quite different from that of galactaric acid. Heating (6 h at 95°C) of the substance from fraction F_2 in 0·1M methanolic hydrogen chloride afforded a solid, m.p. 172—173°C, undepressed on admixture with authentic dimethyl allarate[47]. Identity of both dimethyl ester samples was also established by IR spectrum.

Methanolysis of Thuringiensin (*Ia*)

A mixture of thuringiensin (*Ia*; 71·3 mg) and 0·2M-HCl in methanol (8 ml) was heated in a sealed tube for 8 h at 95°C, allowed to cool, diluted with methanol (20 ml), and neutralised with Dowex 1

TABLE I
Electrophoretic Mobility (cm) of Thuringiensin and Related Compounds in Buffer Solution E_3

Compound	Mobility	Compound	Mobility
Thuringiensin (*Ia*)	6·1	AMP	3·2
Thuringiensin lactone	6·1	ADP	7·4
Dephosphorylated thuringiensin *Ib*	3·05	ATP	8·4

(HCO_3^-) ion exchange resin. The resin was filtered off and the filtrate applied to a column (1 × × 5 cm) of Dowex 50 (H^+) ion exchange resin. Elution of the column with water (50 ml) and evaporation of the eluate under diminished pressure yielded 32·9 mg of a sirup corresponding on chromatography and electrophoresis to fraction F_1 obtained in the preceding experiment. The subsequent elution of the column with 5% aqueous ammonia (50 ml) yielded 6·6 mg of adenine. The Dowex 1 (HCO_3^-) resin which had been used for neutralisation of the reaction mixture after methanolysis, was eluted with 5% aqueous formic acid (50 ml) and the eluate evaporated to afford 25 mg of a sirup. Electrophoretic mobility in the buffer solution E_2: 4·0 cm (weak spot of allaric acid), 15·0 cm (phosphoric acid), 9·5 cm and 10·7 cm (both spots give a positive phosphate test and reduce sodium periodate). Picric acid as standard: 7·0 cm.

Isopropylidene Derivatives of Anomeric Methyl Glycosides *II*

A solution of fraction F_1 (an anomeric mixture of methyl glycosides *II*; 18·9 mg) in dimethylformamide (0·3 ml) was treated with acetone (2·0 ml), 2,2-dimethoxypropane (1·0 ml), and dioxane (0·05 ml) saturated with hydrogen chloride. The whole mixture was kept at 25°C for 3 h, neutralised with triethylamine, and diluted with ethyl acetate (10 ml) to deposit triethylamine hydrochloride which was filtered off and washed with ethyl acetate. The filtrate and washings were combined, taken down under diminished pressure, and the residue coevaporated with two portions of toluene. Chromatography on a column (1 × 25 cm) of silica gel (particle size, 30—50 μm) in the solvent system S_5 (prior to chromatography, the silica gel column was washed with S_5 containing 5% of triethylamine) yielded three fractions (*a*—*c*). The homogeneity of fractions *a*—*c* was checked by thin-layer chromatography in the solvent system S_5: fraction *a* (0·5 mg), R_F 0·21; fraction *b* (the methyl glycoside *IV*; 1·6 mg), R_F 0·38; fraction *c* (1·4 mg), R_F 0·46. The mass spectra of fractions *a*—*c* are almost identical and exhibit at *m/e* 365 a peak corresponding to the M-15 ion in addition to further peaks at *m/e* 333, 317, 262, 230, 213, 205, 199, 173, 141, and 115.

TABLE II

R_F Values and Electrophoretical Mobilities (in cm) of Saccharidic Components of Thuringiensin

Compound	Whatman No 1		Silufol UV_{254}		Electrophoresis[b]
	S_1	S_2	S_3	S_4	E_1
II	0·36	0·37, 0·28[a]	0·40, 0·32[a]	0·36, 0·32[a]	5·2, 3·3[a]
VI	0·18	0·28	0·28	0·22	6·3
VII	0·06	0·15	0·17	0·14	6·9
Allaric acid	0·08	0·02	0·05	0·00	18·3
Dimethyl allarate	0·36	0·37	0·65	0·81	11·7
D-Glucose	0·14	0·20	0·26	0·12	5·5[c]
Methyl β-D-ribofuranoside	0·51	0·53	0·57	0·52	8·8

[a] Weak spot; [b] picric acid, 12·5 cm; [c] diffuse spot.

Acetylation of the Glycoside *IV*

The methyl glycoside *IV* (fraction *b*; 10·5 mg) was dissolved with cooling (bath temperature, 15°C) in a mixture of pyridine (1·5 ml) and acetic anhydride (0·5 ml). The whole was kept at 20°C for 12 h, diluted with ethanol (5 ml), allowed to stand for 10 min, and evaporated under diminished pressure. The residue was coevaporated with two portions of toluene and finally chromatographed on a column (1 × 20 cm) of silica gel in the solvent system benzene–ethyl acetate (2 : 1) to afford 12·1 mg of the sirupous acetyl derivative *V*, homogeneous on thin-layer chromatography in benzene–ethyl acetate (2 : 1). ^{1}H-NMR spectrum (in deuteriochloroform): pentose moiety, H_1 (4·90 s, $J_{1,2} = 0$ Hz), H_2 (4·54 s), H_3 (4·54 s, $J_{3,4} = 0$ Hz), H_4 (4·21, $J =$ = 7·7 Hz), H_5 (3·54, $J = 7{\cdot}7$ Hz); hexose moiety, $H_{1'}$ (4·84, $J_{1',2'} = 3{\cdot}5$ Hz), $H_{2'}$ (4·79, $J_{2',1'} =$ = 3·5 Hz, $J_{2',3'} = 10{\cdot}0$ Hz), $H_{3'}$ (5·48 t, $J_{3',2'} = 10$ Hz, $J_{3',4'} = 9$ Hz), $H_{4'}$ (3·48 dd), $H_{5'}$ (3·78 dd, $J_{5',4'} = 9{\cdot}8$ Hz, $J_{5',6'a} = 3{\cdot}3$ Hz, $J_{5',6'b} = 3{\cdot}3$ Hz), $H_{6'}$ (4·34 d, 2 H, $J = 3{\cdot}3$ Hz); OCH_3 (3·27, 3·37); $OCOCH_3$ (2·05, 2·06, 2·10); $C(CH_3)_2$ (1·45, 1·29). ^{1}H-NMR spectrum (in hexadeuteriobenzene): pentose moiety, H_1 (4·95 s, $J_{1,2} = 0$ Hz), H_2 (4·57 s), H_3 (4·57 s, $J_{3,4} =$ = 0 Hz), H_4 (4·41), H_5 (3·55); hexose moiety, $H_{1'}$ (4·76 d, $J_{1',2'} = 3{\cdot}6$ Hz), $H_{2'}$ (4·92 dd, $J_{2',1'} =$ = 3·6 Hz, $J_{2',3'} = 9{\cdot}8$ Hz), $H_{3'}$ (5·75 dd, $J_{3',4'} = 8{\cdot}85$ Hz, $J_{3',2'} = 9{\cdot}8$ Hz), $H_{4'}$ (3·34 dd, $J_{4',3'} = 9{\cdot}35$ Hz, $J_{4',5'} = 9{\cdot}45$ Hz), $H_{5'}$ (3·67 dd, $J_{5',4'} = 9{\cdot}5$ Hz, $J_{5',6'} = 3{\cdot}5$ Hz), $H_{6'}$ (4·26 d, 2 H, $J = 3{\cdot}5$ Hz); OCH_3 (2·91, 3·03); $OCOCH_3$ (1·61, 1·67, 1·78); $C(CH_3)_2$ (1·15, 1·39). The ^{1}H-NMR spectrum of compound *V* is identical with that of an authentic specimen obtained by an unambiguous synthesis[8].

For purposes of comparison, the ^{1}H-NMR spectrum (in deuteriochloroform) of methyl 2,3,4,6-tetra-O-acetyl-α-D-glucopyranoside (*VIII*) was also measured: H_1 (4·92, $J_{1,2} = 3{\cdot}5$ Hz, $J_{2,3} = 10$ Hz), H_2 (4·88), H_3 (5·47), H_4 (5·04 dd, $J_1 = 9{\cdot}5$ Hz, $J_2 = 10$ Hz), H_5 (3·98 dd, $J_1 = 2{\cdot}5$ Hz, $J_2 = 4{\cdot}75$ Hz, $J_3 = 10$ Hz), H_{6a} (4·07 dd, $J_1 = 4{\cdot}75$ Hz, $J_2 = 12{\cdot}5$ Hz), H_{6b} (4·27 dd, $J_1 = 2{\cdot}5$ Hz, $J_2 = 12{\cdot}5$ Hz); OCH_3 (3·39); $OCOCH_3$ (1·97, 1·99, 2·04, 2·06).

Methyl Glycosides *VI* and their Transformation to 1-Phenylflavazoles *IX*

A mixture of anomeric methyl glycosides *II* (147 mg) was kept in 0·1M-HCl (20 ml) for three days at 25°C, neutralised with Dowex 1 (HCO_3^-) ion exchange resin, the resin filtered off, and the filtrate evaporated under diminished pressure. The sirupous residue of methyl glycosides *VI* (114 mg) was chromatographically homogeneous (Table II) and gave a positive 2,3,5-triphenyltetrazolium chloride test. *o*-Phenylenediamine (76 mg; 0·70 mmol), phenylhydrazine hydrochloride (500 mg; 3·5 mmol), acetic acid (168 mg; 2·8 mmol), and water (3·0 ml) were then added to the above residue (114 mg; 0·35 mmol), the whole heated at 95–100° for 8 h, diluted with 50% aqueous methanol (50 ml), and passed through a column of Dowex 50 (H^+) ion exchange resin. The yellow effluent was chromatographed on a column (1 × 20 cm) of silica gel in the solvent system ethyl acetate–ethanol (5 : 1). Evaporation of the eluate yielded 49·1 mg of the yellow compound *IX*, m.p. 195–200°C. Mass spectrum: *m/e* 482 (M), 288, and 275 corresponding to ions *XXI* and *XXII*.

The 1-phenylflavazole derivative *IX* (41·5 mg) was dissolved in a mixture (1·0 ml) pyridine–acetic anhydride (2 : 1), the solution kept at room temperature for 12 h, and processed analogously to the preparation of compound *V*. Chromatography on a column (1 × 20 cm) of silica gel in ethyl acetate–benzene (2 : 1) yielded 34 mg of compound *X* which was recrystallised from ethanol to afford 24 mg of an analytically pure mixture of anomers *X* (in this mixture, the β-anomer *XI* predominated). M.p. of the anomeric mixture *X*, 175–175·5°C (ethanol). For $C_{32}H_{34}$·N_4O_{11} (650.6) calculated: 59·07% C, 5·26% H, 8·61% N; found: 58·85% C, 5·33% H, 8·75% N. ^{1}H-NMR spectrum (in deuteriochloroform): 1,2-ethanediol portion, H_1 (6·59 dd, $J_1 = 4{\cdot}5$ Hz, $J_2 = 7$ Hz), H_2 (4·47 dd, $J_1 = 7$ Hz, $J_2 = 10$ Hz; 4·27 dd, $J_1 = 4{\cdot}5$ Hz, $J_2 = 10$ Hz); hexose

moiety, $H_{1'}$ (4·57 d, $J_{1',2'} = 8$ Hz), $H_{2'}$ (4·86 dd, $J_{2',1'} = 8$ Hz, $J_{2',3'} = 10$ Hz), $H_{3'}$ (5·21 dd, $J_{3',2'} = 9{\cdot}5$ Hz, $J_{3',4'} = 8$ Hz), $H_{4'}$ (3·71 dd, $J_1 = 8$ Hz, $J_2 = 10$ Hz), $H_{5'}$ and $H_{6'}$ (4·15 to 4·64); OCH_3 (3·46, 3·36); $OCOCH_3$ (2·02, 2·04, 2·16, 2·20). ^{1}H-NMR spectrum (in hexadeuteriobenzene): hexose moiety, $H_{1'}$ (4·01 d, $J_{1',2'} = 8$ Hz), $H_{2'}$ (5·14 dd, $J_{2',1'} = 8$ Hz, $J_{2',3'} = 10$ Hz), $H_{3'}$ (5·34 dd, $J_1 = 9$ Hz, $J_2 = 10$ Hz), $H_{4'}$ (3·49 dt, $J_1 = 9$ Hz, $J_2 = 9$ Hz); OCH_3 (3·10, 2·84); $OCOCH_3$ (1·61, 1·69, 1·86, 1·88).

For purposes of comparison, the ^{1}H-NMR spectra (in deuteriochloroform) were measured of the following model compounds: compound *XV*, H_1 (6·81 t, $J = 5{\cdot}75$ Hz), H_2 (4·93 d, 2 H, $J = 5{\cdot}75$ Hz); $OCOCH_3$ (2·22, 2·05). Compound *XVI*, H_1 (6·77 d, $J = 6{\cdot}0$ Hz), H_2 (6·07 d, $J_1 = 6{\cdot}0$ Hz, $J_2 = 6{\cdot}0$ Hz, $J_3 = 3{\cdot}5$ Hz), H_3 (4·77 d, $J_1 = 6{\cdot}0$ Hz, $J_2 = 12{\cdot}5$ Hz; 4·66 dd, $J_1 = 3{\cdot}5$ Hz, $J_2 = 12{\cdot}5$ Hz); $OCOCH_3$ (2·21, 2·02, 1·99).

Oxidation of Glycosides *VI* to the Anomeric Mixture of Lactones *XII*

Glycosides *VI* (139 mg) were dissolved in a mixture of 0·2M potassium carbonáte and 0·2M sodium hydrogen carbonate (50 ml each), the solution treated with 0·1M iodine in 5% aqueous potassium iodide (30 ml), the whole kept at room temperature for 2 h, and the unreacted hypoiodate reduced by introduction of gaseous sulfur dioxide. The mixture was neutralised with acetic acid (evolution of carbon dioxide), adjusted to pH 9 with 0·1M-NaOH, and applied to a column (1·5 × 15 cm) of Dowex 1 (formate) ion exchange resin. The column was washed with water (50 ml) and then eluted with 10% aqueous formic acid. The eluate was evaporated under diminished pressure to afford 124 mg of a sirup which was dissolved in 80% aqueous formic acid (5 ml). The solution was kept at room temperature for 12 h, evaporated under diminished pressure, and the residue chromatographed on a column (1 × 20 cm) of silica gel in the solvent system S_5 to afford 23·6 mg of compound *XII*. IR spectrum (in KBr): 1760 cm^{-1} (C=O of γ-lactone). ORD spectrum (in water): $[\Phi]_{236.5}$ −2160° (trough). Acetylation of the product *XII* and isolation of the acetyl derivative was carried out analogously to the preparation of the acetyl derivative *V*. ^{1}H-NMR spectrum (in deuteriochloroform) of the acetyl derivative *XIII*: pentose moiety, H_2 (5·61 d, $J_{2,3} = 6{\cdot}5$ Hz, $J_{2,4} = 0$ Hz), H_3 (5·33 dd, $J_{3,2} = 6{\cdot}5$ Hz, $J_{3,4} = 0{\cdot}6$ Hz), H_4 (4·47), H_5 (3·88); hexose moiety, $H_{1'}$ (4·85, $J_{1',2'} = 3{\cdot}5$ Hz, $J_{2',3'} = 10$ Hz, $J_{1',3'} = 0$ Hz), $H_{2'}$ (4·80), $H_{3'}$ (5·50), $H_{4'}$ (3·78 dd, $J_1 = 9{\cdot}4$ Hz, $J_2 = 10$ Hz), $H_{5'}$ (3·84), $H_{6',a}$ (4·70), $H_{6',b}$ 4·14 dd, $J_1 = 4$ Hz, $J_2 = 12{\cdot}5$ Hz); OCH_3 (3·39, 3·48); $OCOCH_3$ (2·05, 2·07, 2·10 (6 H), 2·15).

^{1}H-NMR spectrum of γ-D-ribonolactone (*XVII*) in hexadeuteriodimethyl sulfoxide: H_2 (4·42 d, $J_{2,3} = 5{\cdot}3$ Hz), H_3 (4·13 dd, $J_{3,2} = 5{\cdot}3$ Hz, $J_{3,4} = 0{\cdot}8$ Hz), H_4 (4·24 dt, $J_{4,3} = 0{\cdot}8$ Hz, $J_{4,5} = 6{\cdot}9$ Hz), H_5 (3·59 d, $J = 6{\cdot}9$ Hz); OH_2 (7·75 d, $J = 5{\cdot}66$ Hz), OH_3 (5·26 d, $J = 3{\cdot}8$ Hz), OH_5 (5·1 t, $J = 5{\cdot}5$ Hz).

Hydrolysis of the Dephosphorylated Thuringiensin *Ib* with Trifluoroacetic Acid with the Formation of Compound *VII*

A mixture of compound *Ib* (29·5 mg) and 50% aqueous trifluoroacetic acid (2 ml) was heated at 95°C for 1 h and evaporated under diminished pressure. The residue was diluted with water (15 ml) and neutralised with Dowex 1 (acetate) ion exchange resin. The resin was filtered off and the filtrate treated with Dowex 50 (H^+) ion exchange resin until the UV absorption at 260 nm disappeared. The resin was filtered off and the filtrate evaporated to afford 6·7 mg of the sirupous compound *VII* (for its chromatographic behaviour see Table II). Compound *VII* (3·0 mg) was dissolved in 0·2M-HCl in methanol (5 ml), the solution kept at room temperature for 20 min, and neutralised with Dowex 1 (HCO_3^-) ion exchange resin. The chromatographical and electrophoretical behaviour of thus-obtained glycosides was identical with that of glycosides *II*.

Hydrolysis of the Dephosphorylated Thuringiensin *Ib* (Isolation of D-Glucose)

A solution of compound *Ib* (5·0 mg) in 50% trifluoroacetic acid (0·5 ml) was heated at 95°C for 16 h and processed analogously to the preceding experiment. The mixture was chromatographed on paper Whatman No 3 MM in the solvent system S_1. The band corresponding by its mobility to glucose was eluted, the eluate evaporated, and the residue dried by repeated coevaporations with pyridine. The final residue was then transformed by a reported procedure[48] to the trimethylsilyl derivative, and analysed by gas chromatography. The two main peaks with retention times 31.5 min and 34 min with the peak height ratio of 1·26 correspond to retention times of the trimethylsilyl derivatives of glucose anomers. In another experiment, a portion of the hydrolytical mixture was chromatographed on paper Whatman No 1 in the solvent system S_1. The spot of the R_F value corresponding to D-glucose gave a positive peroxide oxidase test[21].

Methylation of the Dephosphorylated Thuringiensin *Ib*

The free acid *Ib* (68·5 mg; 0·1 mmol) was added to a stirred suspension of sodium hydride (100 mg; 4·1 mmol) in dimethylformamide (10 ml), the stirring continued for 10 min, the mixture treated with methyl iodide (1 ml), stirred for 2 h, and treated with additional sodium hydride (100 mg; 4·1 mmol) and methyl iodide. After stirring for 3 h, the mixture was diluted with chloroform (20 ml), the insoluble portion filtered off, and the filtrate evaporated under diminished pressure. The residue was kept in 0·1M-NaOH (10 ml) for 2 h at room temperature and the alkaline solution applied to a column (1·5 × 10 cm) of Dowex 1 (formate) ion exchange resin. The column was washed with water (50 ml), the product eluted with 10% aqueous formic acid (50 ml), the eluate evaporated, and the residue (88·6 mg) applied to a column (1 × 6 cm) of Dowex 50 (H^+) ion exchange resin. Elution of the column with 5% aqueous ammonia and evaporation of the eluate yielded 20·7 mg of a residue which was dissolved in water (5 ml) and the aqueous solution passed through a column (1 × 2 cm) of Dowex 50 (H^+) ion exchange resin. The effluent was evaporated under diminished pressure, the residue dissolved in methanol (5 ml), and the solution treated with ethereal diazomethane until the yellow colour of the mixture was persistent. The mixture was evaporated and the residue chromatographed on a column (1 × 20 cm) of silica gel in the solvent system ethyl acetate–methanol (8 : 1). Yield, 6·6 mg of the sirupous permethylated derivative *XXIII* (R_F value, 0·57 on thin-layer chromatography in the above solvent system). IR spectrum (in chloroform): 1 750, 1 732 sh cm^{-1} (C=O); 1 632, 1 599, 569 cm^{-1} (purine ring system). UV spectrum (in water): λ_{max} 214 nm and 275 nm; λ_{max} 267 nm (in 0·1M-HCl); λ_{max} 216 nm and 275 nm (in 0·1M-NaOH). Mass spectrum: *m/e* 803 (0·4), 789 (2·5), 774 (2·0), 758 (2·6), 726 (1·5), 686 (4·2), 580 (2·9), 566 (14·6), 553 (3·5), 534 (3·7), 510 (32·7), 478 (13·4), 322 (60·7), 306 (56·7), 220 (27·4) 192 (69·2), 164 (100).

6-Dimethylamino-9-(2,3,5-tri-O-methyl-β-D-ribofuranosyl)purine

A stirred solution of adenosine (621 mg; 3 mmol) in dimethylformamide (25 ml) was treated with sodium hydride (360 mg; 22·5 mmol) and then methyl iodide (3·22 mg; 22·5 mmol) was added over 5 h at room temperature. The whole was kept at room temperature for 12 h, poured into 50 ml of 1% aqueous acetic acid, and the product extracted continuously with chloroform for 36 h. The extract was evaporated and the residue chromatographed on a column of silica gel in the solvent system ethyl acetate–ethanol (3 : 1) to afford a sirup which solidified in the course of several days; yield, 0·662 g. The analytical sample was obtained by sublimation (bath temperature, 80—100°C) at 0·1 Torr; m.p. 69·5—70·5°C. UV spectrum (in 0·1M-HCl): λ_{max} 212 nm and 268 nm (log ε 4·06 and 4·14, resp.); in water: λ_{max} 212 nm and 268 nm (log ε 4·06 and 4·13, resp.);

in 0·1M-NaOH: λ_{max} 275 nm (log ε 4·16). ^{1}H-NMR spectrum (in deuteriochloroform): 6·20 (d, 1 H, $J_{1',2'}$ = 3·25 Hz, $H_{1'}$), 4·26 (dd, 1 H, $J_{2',1'}$ = 3·25 Hz, $J_{2',3'}$ = 4·55 Hz, $H_{2'}$), 4·03 (dd, 1 H, $J_{3',2'}$ = 4·55 Hz, $J_{3',4'}$ = 6·50 Hz, $H_{3'}$), 4·25 (m, 1 H, $J_{4',3'}$ = 6·50 Hz, $J_{4',5'a}$ = 3·0 Hz, $J_{4',5'b}$ = 2·5 Hz, $H_{4'}$), 3·70 (m, 2 H, $J_{5'a,4'}$ = 7·0 Hz, $J_{5'b,4'}$ = 2·5 Hz, $J_{5'a,5'b}$ = 10 Hz, $H_{5'a}$, $H_{5'b}$), 3·58, 3·45, and 3·44 (s, 3 OCH_3), 3·52 (s, $N(CH_3)_2$), 8·17 (s, 1 H, H_2), 8·32 (s, 1 H, H_8). For $C_{15}H_{23}N_5O_4$ (337·4) calculated: 53·40% C, 6·87% H, 20·76% N; found: 53·45% C, 6·88% H, 20·83% N.

Periodic Acid Oxidation of Thuringiensin (*Ia*) and the Dephosphorylated Thuringiensin *Ib*

Sodium periodate (0·05M, 200 µl) was added to an aqueous solution of thuringiensin (*Ia*; 1·61 µmol) and the mixture kept at 25°C under exclusion of light. Aliquots (10 µl each) were withdrawn after 20, 40, 60, 120, 240, and 420 min and the unreacted periodic acid was determined spectrophotometrically[49]. Periodic acid uptake, mol per 1 mol of compound *Ia* (time in min): 1·20 (20), 1·48 (40), 1·68 (60), 1·98 (120), 2·04 (240), 2·16 (420). Compound *Ib* was oxidised analogously. Periodic acid uptake, mol per 1 mol of compound *Ib* (time in min): 2·30 (20), 2·52 (40), 2·91 (60), 3·54 (120), 4·05 (240), and 4·20 (360). The oxidation of adenosine was performed under analogous conditions for purposes of comparison. Periodic acid uptake, mol per 1 mol of adenosine (time in min): 1·01 (20), 1·01 (40), 1·00 (60), and 1·02 (120).

Hydrolysis of the Thuringiensin (*Ia*) Nucleoside Bond

Thuringiensin (*Ia*; 7·0 mg; 10 µmol) was dissolved in 0·1M-HCl (1·1 ml) and ten 0·1 ml portions of this solution were transferred into ampoules which were sealed and heated at 80°C in a steam bath. In intervals of 30 to 60 min, the heating was interrupted, the content of ampoules applied to paper Whatman No 1, and chromatographed in the solvent system S_1. The adenine band was eluted with water and the content of adenine in the eluate determined spectrophotometrically at 260 nm. The hydrolytical rate of adenosine and 9-β-D-glucopyranosyladenine[50] was measured similarly. Halftime of the nucleoside bond hydrolysis $t_{1/2}$ in min: thuringiensin (*Ia*), 154; adenosine, 72·5. Under the conditions stated, only 2·5% of 9-β-D-glucopyranosyladenine was hydrolysed after 300 min.

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