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ELUCIDATION OF THE CHEMICAL STRUCTURE OF BONGKREKIC ACID—I *

ISOLATION, PURIFICATION AND PROPERTIES OF BONGKREKIC ACID

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Abstract—The preparation, isolation and purification of the toxic antibiotic bongkrekic acid (BA), produced by *Pseudomonas cocovenenans* on partially defatted coconut are described. It has been shown that BA is a branched unsaturated tricarboxylic acid with a gross formula of $C_{28}H_{38}O_7$. The presence of three methyl groups, one methoxyl group, one ring system and six double bonds—two isolated and two pairs of conjugated double bonds, both conjugated with a carboxyl group—is proved.

INTRODUCTION

BONGKREKIC acid (BA) is a very toxic antibiotic, produced by *Pseudomonas cocovenans* on partially defatted coconut. Its name has been derived from "bongkrek", a moulded coconut product from Banjoemas (Indonesia). "Bongkrek" is prepared by inoculating partially defatted coconut with *Rhizopus oryzae.* It is consumed daily as a side-dish at the rice table by millions of people in Indonesia.[1] In the past repeatedly serious—often fatal—poisoning occurred after consumption of "bongkrek".[2, 3]

The high mortality rate due to bongkrek-poisoning led Mertens and van Veen to do extensive research in the early thirties. In 1933 they found that in cases of toxic "bongkrek" the mould had been overgrown by a bacterium, which they called "bongkrek bacterium".[4, 5] It is now classified as *Pseudomonas cocovenenans.*[6, 7] This bacterium produces on partially defatted coconut two highly toxic compounds.[8–12]

A. *Toxoflavin,* a yellow crystalline product (m.p. 171°) which is very toxic. The LD_{50} for mice after injection is 1·7 mg per kg of bodyweight. By mouth, toxoflavin has a LD_{50} for mice of about 8·4 mg per kg of bodyweight. The chemical structure of toxoflavin was elucidated by van Damme.[11] Its synthesis has been worked out by Daves[13] and some work regarding the mode of action of toxoflavin has been done by Lathuasan[12] and studies on its biosynthesis have been carried out by Levenberg.[14–16]

B. *Bongkrekic acid,* an unsaturated fatty acid was isolated by Mertens and van Veen from toxic bongkrek as a mixture of fatty acids—free of toxoflavin—which nevertheless proved very toxic. They called the toxic principle of this mixture bongkrekic acid. However, they were unable to purify BA and the formulae $C_{11}H_{16}O_3$ and $(C_{11}H_{16}O_3)_2$, suggested by van Veen in 1933[17] and in 1950[18] respectively, were based on impure products. It was only in 1956 that Nugteren[6, 19] improved the isolation and purification. He described BA as a branched unsaturated tricarboxylic acid with

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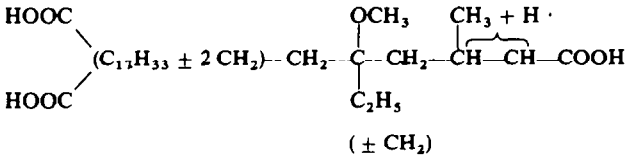
the formula: $C_{29}H_{40}O_7 \pm 2CH_2$. The presence of three carboxyl groups, one methoxyl group and seven double bonds was demonstrated. From cleavage reactions he proposed this partial structure for hydrogenated BA:



Preparation, isolation and purification of BA

BA has been prepared and isolated by the method described by Nugteren[6, 19] with some improvements. As distinct from this method, the ethereal solution of the mixture of fatty acids—thus obtained—was separated by TLC (Fig 1).

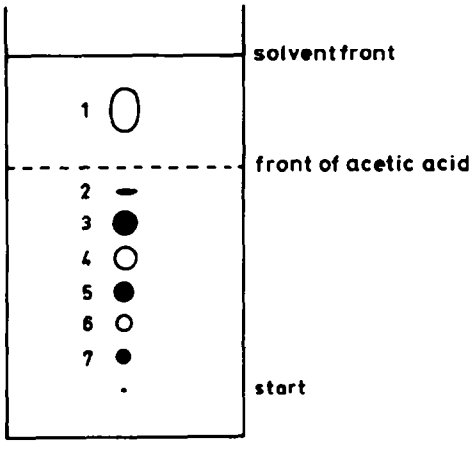


FIG 1. Thin-layer chromatogram of "crude BA-preparations" on silica GF_{254} (Experimental)

As BA has strong antibiotic activity[18] we have been able to determine which spot on the chromatogram corresponded to BA. The spots were scraped off the chromatogram and subsequently tested on the *Cladosporium cucumerinum* mould. Only spot number three showed antibiotic activity. Later on it appeared that this product is very toxic for mice and rats.

Using chromatoplates with a thickness of 1 mm we were able to perform this chromatography on a preparative scale. The BA-band was scraped off from the plate and BA was eluted by means of methanol. The final purification was done by counter current distribution, using the system 0·5 molar phosphate buffer $pH = 6{\cdot}38$/di-n-butyl ether. After 80 transfers BA could be obtained from the fractions 26 to 46 as a colourless oil, which solidified to a white amorphous product with a melting traject of 50–60°. We could not obtain BA or one of its derivatives in a crystalline state. The purity of BA is ascertained by TLC. The substance showed only one sharp spot on the chromatogram with two different eluents: propanol:ammonia (3:1) $R_f = 0{\cdot}4$ and chloroform:methanol:acetic acid (94:5:1) $R_f = 0{\cdot}7$.

Some properties of bongkrekic acid

As ammonia-salt in water BA absorbs strongly in the UV region with two maxima at 239 mμ (ε = 40600) and 263 mμ (ε = 40600) and a minimum at 250 mμ (ε = 37600). A methanolic solution of BA shows two absorption maxima at 237 mμ (ε = 32000) and 267 mμ (ε = 36700) and a minimum at 249 mμ (ε = 28000).

The optical rotation of BA was shown by van Veen and Mertens,[17] but their preparations were not pure. The same objections have been raised against Nugterens' data. The ORD spectra of BA and HBA (hydrogenated BA) were measured with a spectropol-1-spectropolarimeter. BA showed a positive Cotton effect ORD curve with two extrema: a peak at 286 mμ and a trough at 241 mμ. The two points λ_0 = 232 and 260 mμ correspond roughly to the wavelengths of the UV absorption maxima. HBA showed a positive plain ORD curve. The specific optical rotation was determined in a Perkin–Elmer spectropolarimeter—P 141. Table 1 shows the results.

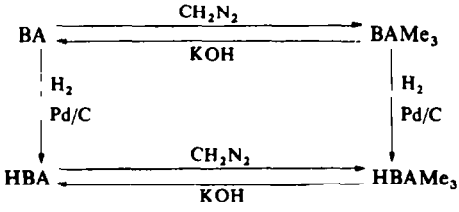
TABLE 1

	α^{25}		
λ in mμ	BA	HBA	
589	+ 162·5°	+ 6·7°	
578	+ 171.5°	+ 6·5°	
546	+ 202·1°	+ 7·6°	accuracy: ± 0·2°
536	+ 430·7°	+ 12·4°	
365	+ 966·7°	+ 18·5°	

BA shows strong antibiotic activity, especially towards moulds and yeasts. In the past some work on the pharmacological activity of BA[20–22] has been done, but the results of this must now be considered out of date. Preliminary investigations on the intravenous toxicity of BA to mice resulted in: LD_{50} = 1·41 mg/kg mice and MED_{50} = 0·0562 mg/kg mice.

BA can be esterified to trimethyl bongkrekate, which shows *one* spot with R_f = 0·6 on a chromatogram of Silica GF_{254}, using cyclohexane:ether as an eluent (1:1). Hydrolysis of the ester with KOH resulted in BA.

By catalytic hydrogenation BA was converted into the very stable hydrobongkrekic



Scheme: a

acid (HBA). From Scheme a we see that $HBAMe_3$ can be prepared from BA in two different ways. The analytical methods applied showed that the end product as well as the intermediates are single compounds. This can be considered a strong argument in support of the purity of BA.

Determination of the functional groups in bongkrekic acid

From the parent $m/e = 528{\cdot}3083$ in the mass spectrum of trimethyl bongkrekate we know the molecular weight and gross formula of $BAMe_3$: $C_{31}H_{44}O_7$. By titration it could be determined that the equivalent weight of BA was 160. So BA must contain three carboxyl groups per molecule. This is in complete accordance with the three acid protons found in the NMR spectrum of BA ($\delta = 10{\cdot}4$ ppm). The three ester peaks ($\delta = 3{\cdot}67$; $3{\cdot}70$ and $3{\cdot}75$ ppm) in the NMR spectrum of $BAMe_3$ indicate that the three COOH groups in BA are structurally not equivalent.

The NMR spectrum of $HBAMe_3$ showed just *one* ester peak ($\delta = 3{\cdot}67$ ppm). In the IR spectra the two ester carboxyl peaks in $BAMe_3$ (1705 and 1735 cm^{-1}) are changed into *one* ester carboxyl peak in $HBAMe_3$ (1735 cm^{-1}). So we may conclude that there are two carboxyl groups in BA conjugated with—or are very near to—double bonds and one carboxyl group without any conjugation.

Catalytic hydrogenation of BA with 10% Pd-C provided the very stable hydrobongkrekic acid (HBA). From the H_2 uptake during hydrogenation and from the difference in molecular weight of $HBAMe_3$ (540) and $BAMe_3$ (528), known from mass spectrometric data, it is clear that BA possesses six double bonds.

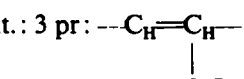
The UV absorption spectrum of BA (as NH_4-salt in H_2O) shows maxima at 239 and 263 mμ. Using the rules of Woodward[23,24] we have been able to obtain indications for the presence of two independent systems of conjugated double bonds in BA. However, we were unable to produce Diels–Alder adducts. Also, Br_2-addition occurred incompletely: four atoms of bromine were rapidly absorbed and after this the bromine-uptake went on very slowly until a number of 5·25 atoms of bromine per molecule BA had reacted. So we concluded that the two conjugated systems in BA are heavily substituted. The said experiments can be explained by the assumption that in BA two isolated double bonds (which have been brominated rapidly) and four double bonds, conjugated in two pairs are present, which are strongly hindered by substituents.

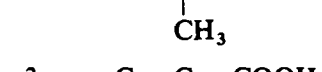
BA contains one OMe group, as appeared from a determination of methoxyl according to Zeisel, which resulted in four Me groups per molecule $BAMe_3$ (three $COOCH_3$-groups and one OMe group). The ether group is also significant in the NMR ($\delta = 3{\cdot}26$ ppm in BA; $\delta = 3{\cdot}37$ ppm in HBA) and IR spectra (1105 cm^{-1} in $BAMe_3$ and 1100 cm^{-1} in $HBAMe_3$). The difference in resonance between the OMe groups in the NMR spectra of BA and HBA indicates a double bond in the neighbourhood of the OMe group in BA.

The totally hydrogenated HBA no longer contains C=C double bonds. Attempts to increase the hydrogen uptake fail. There are no vinyl resonances in the NMR spectrum of HBA, and the UV spectrum of HBA does not show an absorbancy above 200 mμ. The gross formula of HBA, $C_{28}H_{50}O_7$, indicates the presence of a ring system in the molecule. Replacement of the three carboxyl groups and the OMe group by hydrogen results in $C_{24}H_{48}$. This formula must contain a ring system.

In the NMR spectrum of BA we see three Me resonances, which indicate the presence of the following groups in BA:

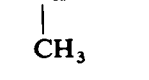
$\delta = 1{\cdot}08$ ppm (doublet) Int.: 3 pr: —$\underset{\displaystyle CH_3}{\overset{\displaystyle |}{C_H}}$—

δ = 1·88 ppm (singlet) Int.: 3 pr: 

δ = 1·95 ppm (singlet) Int.: 3 pr: 

The NMR spectrum of HBA shows the presence of three OMe groups in HBA:

δ = 0·87 ppm (doublet) = 6 Hz Int.: 6 pr: 2x 

δ = 1·13 ppm (doublet) = 7 Hz Int.: 3 pr: 

CONCLUSIONS

BA is an unsaturated, branched tricarboxylic acid with a gross formula of $C_{28}H_{38}O_7$ (mol weight: 486). The presence of three carboxyl groups, three Me groups, one OMe group, one ring system and six double bonds per molecule has been proved. We have been able to demonstrate the presence of two isolated double bonds and two pairs of conjugated double bonds (both conjugated with a carboxyl group).

EXPERIMENTAL

Pseudomonas cocovenans. The BA-producing microorganism was present in the laboratory in the form of lyophylized cultures, prepared by Nugteren.[9] These cultures showed a constant BA production, when they were kept on 1% glycerol—1% pepton agar slants.

Preparation of bongkrekic acid. We prepared BA as described by Nugteren[6] with some improvements. 2·5 kg of commercial copra was twice defatted with water of 80°. The partially defatted coconut was sterilized at 110° for 20 min. The product (1·5 kg; pH = 6·6) was divided into twelve pans (diam 20 cm; height: 10 cm). Each pan was subsequently inoculated with 25 ml of a 16 hr old culture of *Pseudomonas cocovenenans* on 1% peptonwater − 2% glycerol. After an incubation of 4 days—during which time the contents of each pan was stirred well daily—at 30° and a relative humidity of 70%, the toxic material was allowed to dry at 30° (two days). The production of BA was tested using the antibiotic activity of BA towards *Cladosporium cucumerinum.*[6]

The dry material (pH = 5) was extracted with light petroleum (60–80°), after which the light petroleum extract (about 3·5 l.) could be extracted by an 8% soln $NaHCO_3$ aq (7 × 50 ml). This soln was acidified to pH = 2 and extracted with 3 × 50 ml ether. The ethereal soln, washed with water, was extracted with three portions of 2N ammonia (25 ml). In this way we obtained a mixture ("crude BA mixture") of fatty acids (75 ml) in 2N ammonia among which about 2·5 gram of BA. (measured spectrofotometrically).

Thin layer chromatography. The crude BA-mixture was separated by TLC on silica GF_{254} (Merck) with a mixture of $CHCl_3$, MeOH and AcOH as a solvent, in a ratio of 94:5:1. R_f of BA: 0·7.

The mixture was extracted with ether and the ethereal soln spotted on the chromatogram.

The unsaturated compounds were visible in UV light as dark spots on the chromatoplate. All acids could be demonstrated by spraying with a bromocresolgreen soln in EtOH and exposing the plate to ammonia (the acids form yellow spots on a blue background). The unsaturated compounds could also be detected by spraying with a saturated soln of $SbCl_3$ or by spraying with alkaline-$KMnO_4$ soln.

TLC systems used:

Substance	absorbent	solvent		detection
BA	Silica GF_{254}	chloroform 94 methanol 5 acetic acid 1	A	UV, J_2-vapour 0·04% bromocresol-green methanol followed by 0·5% $KMnO_4$ in 2n-soda
BA	Silica GF_{254}	propanol 3 ammonia 1	B	idem
HBA	Silica GF_{254}	A and B		J_2-vapour or spray of 0·04% bromocresolgreen
$BAMe_3$	Silica GF_{254}	cyclohexane 1 ether 1		UV, J_2-vapour
$BAMe_3$	Silica— 5% $AgNO_3$	cyclohexane 1 ether 2		0·02% dichloro-fluoresceine in ethanol
$HBAMe_3$	Silica GF_{254}	cyclohexane 1 ether 1		J_2-vapour

The preparative TLC was performed in the usual way. The BA band (visible under UV-light $\lambda = 254$ mμ) was scraped off the plate as described by Ritter.[26] BA was eluted from the silicagel with MeOH. After filtration through a Seitz filter the methanolic soln was evaporated with a rotating disc evaporator. The residue, a yellowish oil, was called "chromatographically pure BA". Yield: 130 mg of dry weight of the crude BA mixture was purified on one chromatoplate and yielded about 30 mg of chromatographically pure BA.

Counter current distribution. The CCD was carried out in a Von Metzsch apparatus[25] using the system di-n-butyl ether as the upper phase and 0·5 molar phosphate buffer ($pH = 6·38$) as the lower phase. In each run we purified about two grams of "chromatographically pure BA".

After 80 transfers the fractions 26 to 46 proved to contain BA (determined by TLC). After collection of the upper phases of tubes 26 to 46 the di-n-butyl ether soln was extracted with 2N ammonia. The 2N ammonia soln was subsequently extracted with peroxide-free ether.

The lower phases were acidified with H_3PO_4 to $pH = 1$ and extracted with peroxide-free ether as well. The combined ethereal extracts (washed with water and dried with Na_2SO_4) were evaporated with a rotating evaporator.

The residue (a colourless oil) was dried on KOH, P_2O_5 and paraffin chips in a vacuum desiccator. BA solidified to a white amorphous product with a melting traject of 50–60°, yield after CCD was about 90%; Found C, 68·00; H, 8·15. (Calc. for $C_{28}H_{38}O_7$: C, 69·12; H, 7·82%).

Preparation of $BAMe_3$ *and* $HBAMe_3$. BA and HBA were esterified with diazomethane or by refluxing for 2 hr with MeOH/5% HCl/5% dimethyl carbonate. (Found: ($BAMe_3$): C, 69·84; H, 8·13. Calc. for $C_{31}H_{44}O_7$: C, 70·44; H, 8·33%).

Hydrogenation of BA. BA was hydrogenated with 10% Pd-C as catalyst. The hydrogenation occurred fast (600 mg BA in 30 to 40 min) when performed in H_2O as the NH_4-salt or in a methanolic soln. (Found (HBA): C, 67·45; H, 10·23. Calc. for $C_{28}H_{50}O_7$: C, 67·47; H, 10·04%).

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