PREPARATION OF ^{3}H-LABELLED BONGKREKATE

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1. Introduction

Bongkrekic acid (BKA) has turned out to be an extremely useful tool for research on the ADP, ATP carrier function [1–3]. In particular in the research on the binding to the ADP, ATP carrier sites in the membrane, bongkrekate was instrumental in elucidating the reorientation mechanism for the translocator function [4–7]. These studies were based on using radioactively labelled ADP and ATP in the ^{3}H- or ^{14}C-labelled form and ATR and CAT in the ^{35}S-labelled form. It was therefore highly desirable, also to obtain radioactively labelled BKA*.

Attempts to produce radioactively labelled BKA by biosynthesis analogous to ATR, had no success in producing BKA in yields sufficient for systematic binding studies (Klingenberg, unpublished) [8]. For this purpose the BKA producing organism *Pseudomonas cocovenenans* was grown on ^{14}C-labelled glucose or other compounds.

In the present communication the synthesis of ^{3}H-labelled BKA with high specific activity and with high yield is described, using a relatively simple procedure by exchanging activated hydrogen against ^{3}H derived from ^{3}H-H_2O. The identity of the ^{3}H-labelled compound with the original BKA will be documented both from the chemical and biological point of view.

2. Materials and methods

Bongkrekic acid at about 80% purity was a gift by Professor W. Berends, Delft. ^{3}H-H_2O (5 Ci/ml and 200 mCi/ml) was purchased from Amersham. D_2O (99.7%) was obtained from Merck, Sharp and Dohme, and $CDCl_3$ (99.7%) from Merck.

Preparation of [^{3}H]BKA: 100 mg 80% pure BKA were dissolved in 5 ml diethyl ether. At room temperature freshly prepared diazo-methane solution was added dropwise until the solution turned yellow. This solution was stored overnight in the refrigerator for completing the reaction. After the solvent was removed by evaporation, the residue was dissolved in methanol. The concentration of the BKA-trimethyl-ester ($BKMe_3$) was determined by the absorption spectrum which has a maximum (at 270 nm), slightly shifted to higher wavelength from the free BKA [9].

The one step ^{3}H incorporation and hydrolysis of the ester was carried out by solving 25 mg $BKMe_3$ corresponding to 50 μmol in 800 μl dioxane under addition of 5 equivalents NaOH and 400 μl of ^{3}H-H_2O. The ^{3}H-H_2O was sublimated into the reaction vessel on a high vacuum line with a diffusion pump to minimize contamination. The hydrolysis was performed at 40°C for 3 h. The unreacted ^{3}H-H_2O was sublimated back together with the dioxane. The residual yellowish oil was dissolved in 1 ml H_2O and acidified with 130 μl 2 N HCl. ^{3}H-BKA was extracted three times with 0.5 ml diethylether. The combined extracts were lyophilized. In order to remove the ^{3}H in the COOH groups the [^{3}H]BKA was dissolved in 1 ml of methanol and again lyophilized. The purification was performed on preparative thin-layer chromatography, 0.5 mm silica-gel (plates) with chloroform: methanol: acetic acid = 94 : 5 : 1. About 30% of the starting material was recovered as [^{3}H] BKA. A spec. act. was determined as 158 000 dpm/

*Abbreviations: BKA, bongkrekic acid; $BKMe_3$, BKA-trimethyl-ester; CAT, carboxy-atractylate.

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nmol on the basis of the absorption spectrum.

The kinetics of $BKMe_3$ hydrolysis was followed by dissolving 5 mg of $BKMe_3$ in 100 μl dioxane and 50 μl ^3H-H_2O. The other conditions were the same as described above. The reaction was started by addition of NaOH and at each time, indicated in fig.3, 5 μl samples were removed and acidified by 5 μl 1 N HCl. The samples were extracted with ether, lyophilized and treated for removal of labile ^3H as mentioned above. Aliquots of the samples were then applied to thin-layer chromatography, with original BKA and $BKMe_3$ as references. Another aliquot was used for determining the total counts. The ultraviolet visible spots were eluted with methanol and counted.

For the synthesis of deutero-BKA the same procedure as described above was applied with the only exception that D_2O instead of ^3H-H_2O was used in the mixture with dioxane as solvent.

For the n.m.r. spectra pure BKA and deutero-BKA were assayed at 90 MHz, 10 mg of the substances were dissolved in 400 μl $CDCl_3$. Internal standard was tetramethylsilane.

The inhibition of the ADP transport by BKA was assayed by the 'back-exchange' method [11]. For this purpose rat liver mitochondria were prelabelled with [^{14}C]ADP and washed.

3. Results

The strategy for the ^3H incorporation was based on the concept that the structure [10] between the two carboxylic groups at C_1 and C_{24} position can be considered as a vinylogic homologue of malonic acid (fig.1). Therefore, it can be expected that the two hydrogens at C_{23} are relatively acidic and exchangeable against ^3H-H_2O. Exploratory experiments showed, however, that the unmodified BKA does not incorporate ^3H when exposed to ^3H-H_2O for several hours at 60°C

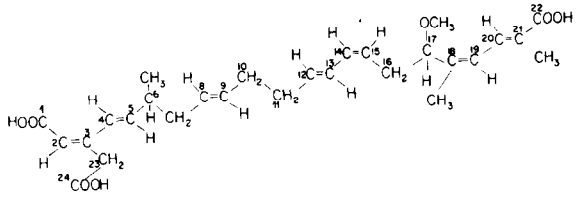


Fig.1. Structure of bongkrekic acid (BKA) (Lijmbach 1973).

in 0.2 M NaOH. Stronger activation of the hydrogens at the 23-methylene position can be achieved by esterification of the carboxyl groups. Therefore, the trimethylester of BKA ($BKMe_3$) was synthesized, the starting material for the exchange against ^3H-H_2O.

In the first stage the ^3H incorporation into the $BKMe_3$ was explored.

$$BKA \xrightarrow{CH_2N_2} BKMe_3 \xrightarrow{NaOMe}$$

$$BKMe_3 \cdot Na \xrightarrow{^3H \cdot H_2O} {}^3H \cdot BKMe_3$$

On the reacting the ester with sodium methylate in methanol, the activated hydrogens were replaced by sodium. The equilibrium was shifted towards the products by removing the methanol in vacuum. On suspending the residue in ^3H-H_2O, ^3H replaced rapidly Na. After a few minutes the suspension was acidified by HCl and the labelled ester could then be extracted by ether. This extract was analyzed by thin-layer chromatography as shown in fig.2. All the activity was concentrated in a peak which moved identical with the original trimethylester.

The formation of the ^3H-ester can also be achieved in a one-step reaction when the ester is exposed under mild alkaline conditions with triethylamine and ^3H-H_2O. After 24 h the ester is quantitatively converted into the ^3H-form. The ^3H-labelled ester can be hydrolyzed to the free [^3H]BKA on addition of five equivalents of NaOH. Besides free [^3H]BKA a by-product is formed.

After it was clear that $BKMe_3$ can easily be labelled with ^3H by exchange in ^3H–H_2O, a simplified one

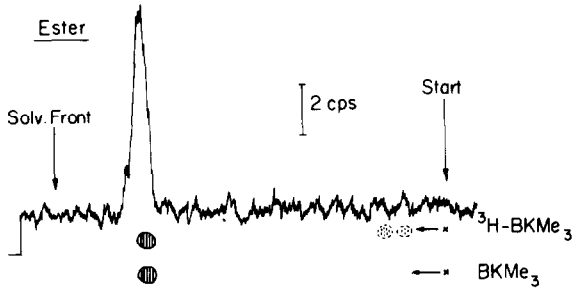


Fig.2. Thin-layer chromatography of the [^3H]trimethylester of bongkrekic acid ($BKMe_3$) on silica-gel (thickness 0.25 mm) developing medium: $CHCl_3$: MeOH: HOAC = 94 : 5 : 1, time: 45 min.

batch procedure was developed. From the results with the ester it can be expected that the exchange against ^3H is considerably faster than the subsequent hydrolysis under appropriate conditions.

$$BKA \xrightarrow{CH_2N_2} BKMe_3 \xrightarrow[\text{fast}]{^3H\cdot H_2O + NaOH} (^3H\cdot BKMe_3) \xrightarrow[\text{slow}]{} {}^3H\cdot BKA \text{ (+ by-product)}$$

Therefore, it should be possible to combine both reactions in one batch for the preparation of [^3H] BKA. The reaction sequence in the one batch process is analyzed by following the kinetics of the combined ^3H-incorporation and hydrolysis of the ester as shown in fig.3. In the first sample, 10 min after starting the reaction, ^3H is found only in the ester form, which is already partially hydrolyzed. The ^3H-incorporation of $BKMe_3$ was obviously completed at a considerably shorter time, not resolved here. [^3H]BKA is generated after about 20 min simultaneously with a by-product. After about 1.5 h all the esters have disappeared and all ^3H is found in BKA and the by-product.

On the basis of these kinetics, the synthesis of [^3H]BKA was repeated with ^3H-H_2O of higher specific activity on a preparative scale. According to the method of the combined reaction steps, $BKMe_3$ was exposed to ^3H-H_2O for 3 h, as described in more detail in methods. For separating the by-product from BKA, thin-layer chromatography was performed as shown in fig.4. The extracted pure [^3H]BKA was then rechromatographed in order to assay its purity.

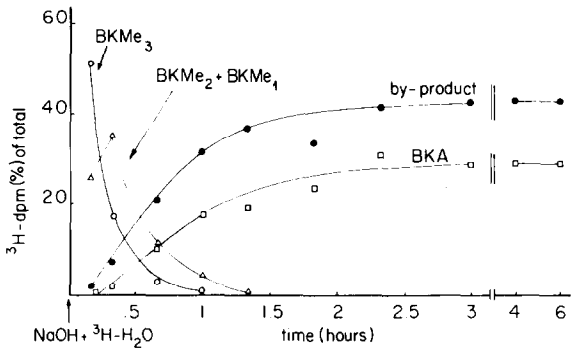


Fig.3. Kinetics of the 'one batch' ^3H incorporation and hydrolysis of $BKMe_3$ at 40°C. For further details see Materials and methods.

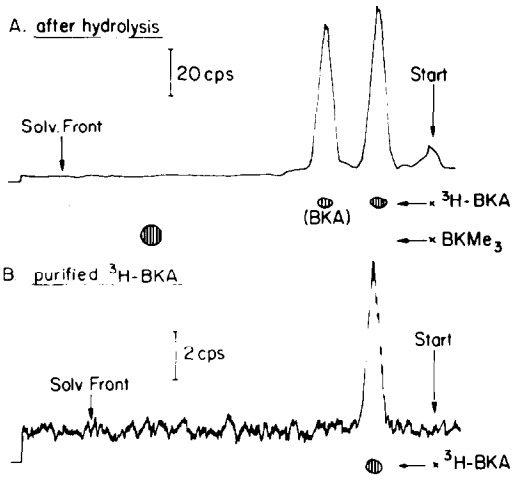
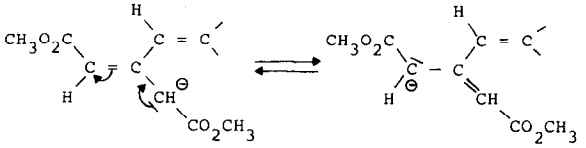


Fig.4. (A) Thin-layer chromatography of the hydrolyzed products of $BKMe_3$ on silica-gel. (B) Thin-layer chromatography of the purified BKA.

The position of the substitution was determined by n.m.r. spectroscopy. For this purpose deutero BKA was prepared according to excatly the same procedures as [^3H]BKA. The n.m.r. spectrum of the deutero-BKA was compared with that of untreated pure BKA as shown in fig.5. In the n.m.r. spectrum of the deutero-BKA, the signals at 3.42 and 3.32 ppm disappeared. These have been assigned by Lijmbach to H at the C-23 position. In addition one signal disappeared at 5.81 ppm which can be assigned to H at C-2 position. This can be explained by an allylic rearrangement which activates this H.



As a result three H are replaced in one molecule BKA. The spectrum further demonstrates that the reaction product of the esterification, subsequent substitution and hydrolysis, is chemically identical with the original compounds. It can be visualized that as a result of the allylic rearrangement a cis-isomer is generated between C_1 and C_2 from the trans-configuration in the original BKA. It might be possible that the by-product obtained after hydrolysis of the ester represents this isomer.

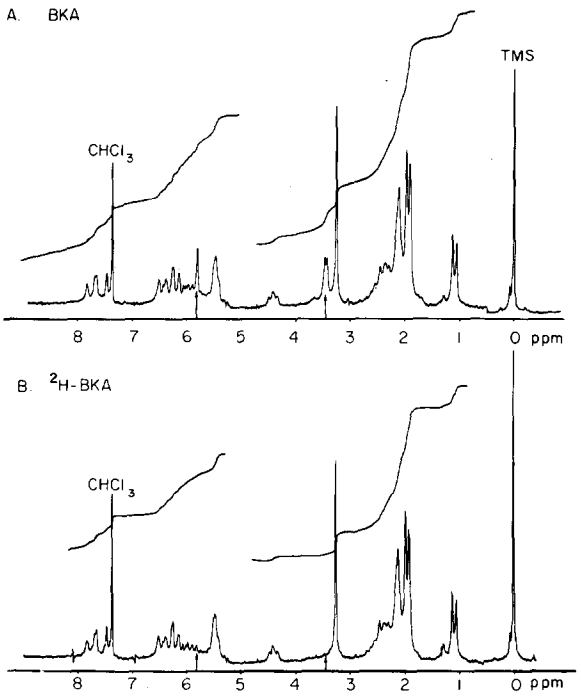


Fig.5. 90 MHz n.m.r. spectra (Bruker SXP 4–100) of (A) 10 mg BKA in 400 μl $CDCl_3$, (B) 10 mg deuterium exchanged BKA in 400 μl $CDCl_3$, with tetramethylsilane (TMS) as an internal standard.

It appeared important to verify that [^{3}H]BKA despite the chemical treatments for the introduction of ^{3}H, has the same biological activity as the original BKA. The biological assay can be considered to be the most sensitive test for structural alterations. The biological activity of [^{3}H]BKA was compared with untreated BKA using a quantitative assay of the inhibition of the adenine nucleotide exchange. For this purpose the 'back-exchange' in rat liver mitochondria was titrated with increasing amounts of [^{3}H]BKA and of BKA as shown in fig.6. Over the whole range the inhibition by [^{3}H]BKA agrees with that of the untreated BKA. From these results it can be concluded that [^{3}H]BKA has virtually the identical structure.

Acknowledgements

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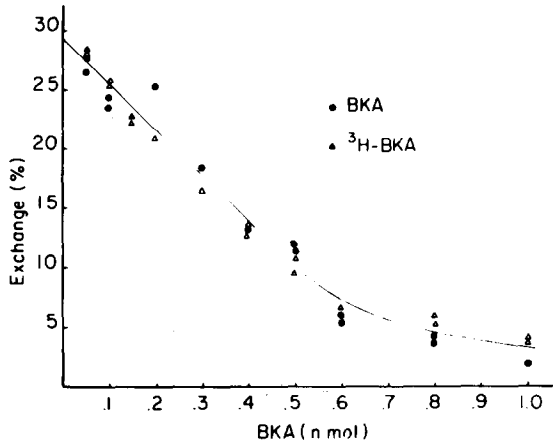

Fig.6. Comparison of BKA and [^{3}H]BKA with respect to the inhibition of the exchange activity of rat liver mitochondria. [^{14}C]ADP prelabelled mitochondria are incubated for 4 min in the presence of increasing amounts of BKA and [^{3}H]BKA at 10°C, 0.25 M sucrose at pH 6.8. The 'back-exchange' is started on addition of 50 μM unlabelled ADP. The exchange was stopped after 10 sec by the addition of 50 μM CAT. After centrifugation the supernatant was assayed for [^{14}C]ADP. The protein content was 0.82 mg/ml.

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