1,4-Thiomorpholine-3,5-dicarboxylic acid, a novel cyclic imino acid detected in bovine brain

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Gas-liquid chromatography of enriched bovine brain extract revealed the occurrence of several sulfur-containing compounds. By co-chromatography with authentic product and by mass-spectrometric analysis, one of these compounds has been identified as 1,4-thiomorpholine-3,5-dicarboxylic acid (TMDA). The possible derivation of TMDA from lanthionine is discussed. This represents the second S-containing cyclic imino acid so far discovered in a mammalian brain whose physiological significance has not yet been explored.

1,4-Thiomorpholine-3,5-dicarboxylic acid *Brain* *Cyclic imino acid* *Sulfur compound* *Lanthionine*

1. INTRODUCTION

Either oxidative [1,2] or transaminative [3,5] deamination of L-cystathionine has been found to yield the mono-keto derivative which spontaneously cyclizes into the internal ketimine form [6]. L-Cystine, L-lanthionine and L-aminoethylcysteine behave similarly [1,6]. The reduced form of cystathionine ketimine, simply named cyclothionine, has been detected in the extracts of bovine brain analyzed by gas-mass spectrometry [7]. Gas-liquid chromatography of bovine brain extracts, using a flame photometric detector for sulfur, revealed the presence of several sulfur-containing compounds [7] worthy of investigation. Here we present evidence for the identification of one of these compounds as the cyclic imino acid 1,4-thiomorpholine-3,5-dicarboxylic acid (TMDA), i.e., the reduced form of lanthionine ketimine (see fig.1).

Abbreviations: TMDA, 1,4-thiomorpholine-3,5-dicarboxylic acid; cyclothionine, 1,4-hexahydrothiazepine-3,5-dicarboxylic acid

2. EXPERIMENTAL

2.1. *Materials*

Bovine brains including cerebellum and brain stem were obtained from the local slaughterhouse and worked out as soon as possible. TMDA was

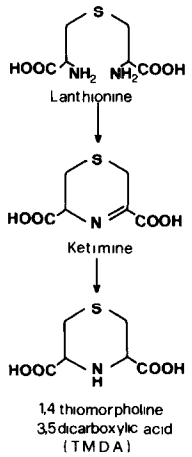

Fig.1. A possible route for the derivation of TMDA from lanthionine. An alternative route is the formation of the ketimine through the β-replacement of cysteine or serine using mercaptopyruvate as substituent.

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prepared by interacting L-cysteine with Br-pyruvic acid to yield lanthionine ketimine [8] which was then reduced by $NaBH_4$ as reported earlier [9]. The isomer 1,3-thiomorpholine-2,4-dicarboxylic acid was prepared by interacting DL-homocysteine with glyoxylic acid (to be published). Other products were of the best commercial source.

2.2. *Enrichment procedure*

Owing to the low content of TMDA it was found necessary to submit the brain extracts to an enrichment procedure before the analyses. The procedure was identical to that described for the enrichment of cyclothionine [7]. In a preliminary experiment 5 mg authentic TMDA were added to a brain homogenate and extracted as indicated in [7]. The final extract was subjected to quantitative gas-liquid chromatography [10] which indicated a total recovery of the added compound. The procedure reported for cyclothionine was thus found suitable also for the enrichment of TMDA.

2.3. *Identification procedures*

Gas-liquid chromatography of synthetic TMDA and related compounds has been described [10]. In this study we made use of a Perkin-Elmer Sigma 300 chromatograph provided with a flame photometric analyzer for sulfur. The 120 cm × 2 mm i.d. glass column was packed with 10% OV-17 on Chromosorb W HP DMCS 100–120 mesh. Temperatures were 200°C for the column, and 220°C for the injector and the detector. Flow rates were 25 ml/min for N_2 carrier gas, 65 ml/min for H_2 and 110 ml/min for air. Gas-chromatographic–mass spectral analyses were performed on a Pye Unicam gas chromatograph connected to an LKB 2091 low-resolution mass spectrometer equipped with a PDP 11 digital computer. Chromatographic separation was carried out on a 25 × 0.2 mm i.d. fused silica capillary column with OV-101 as stationary phase. The flow rate of helium carrier gas was 0.6 ml/min. The column temperature was programmed from 120 to 250°C at a rate of 8°C/min; injector temperature was 250°C; the molecular separator temperature was 260°C. Mass spectrometer experimental conditions were: ionization mode, E.I.; electron energy, 70 eV; ion source, 250°C; ion source vacuum, 0.5×10^{-6} mmHg.

3. RESULTS

Fig.2 shows the gas liquid chromatogram of the final brain extract corresponding to approx. 100 g bovine brain. It shows the presence of one large peak followed by a shoulder exhibiting the same retention times as authentic TMDA. It also shows the presence of the 2 peaks of cyclothionine (peaks b), with a longer retention time as identified earlier [7]. The double peak of TMDA (in the present case, a peak and a shoulder) is due to the dimethyl ester (the large peak) and to the trimethyl derivative (the shoulder), the latter produced by the partial methylation of the imino nitrogen by the diazomethane reagent as reported [10]. The isomer 1,3-thiomorpholine-2,4-dicarboxylic acid exhibited a different elution time and was eliminated as a possible candidate for the identification of peak a.

The main peaks of the endogenous and synthetic TMDA were submitted to mass spectrometry giv-

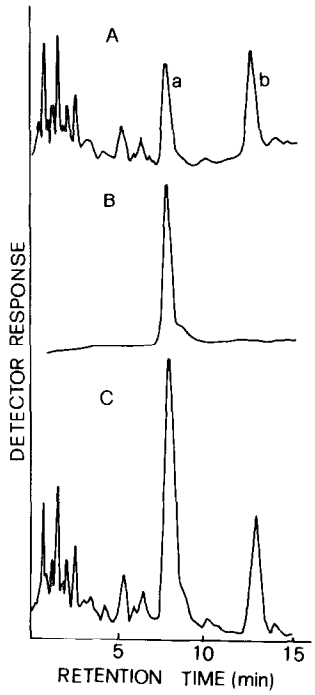

Fig.2. Gas-liquid chromatography using a flame photometric detector for sulfur of enriched bovine brain extract. See text for details. A, bovine brain extract. B, 1 μg authentic TMDA. C, A + B. Peaks: a, TMDA; b, cyclothionine. The sample of brain extract used for A and C corresponds to approx. 100 g wet brain.

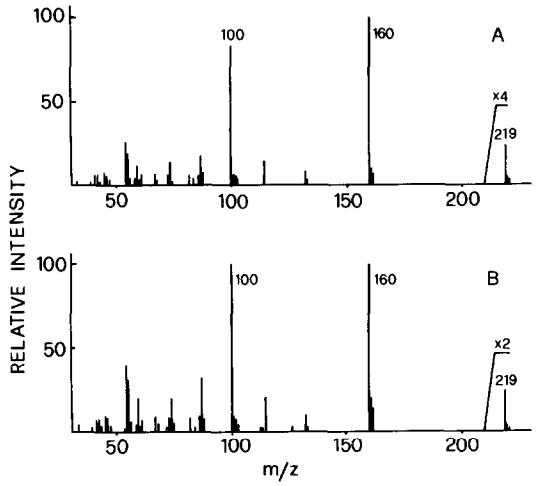

Fig.3. Mass spectrum of the main peak of bovine brain TMDA, separated as indicated in fig.2A, compared with the mass spectrum of the same peak of authentic TMDA, separated as in fig.2B. m/z = 219 is M^+, the molecular ion; m/z = 160 is M^+ less -$COOCH_3$; m/z = 100 is M^+ less 2 -$COOCH_3$, less H.

ing the fragmentation patterns in fig.3. The molecular ion M^+ (m/z = 219), accompanied by the natural isotopic congeners $M^+ + 1$ and $M^+ + 2$, is evident in both patterns. The fragmentations are very similar giving an overall superimposable picture. Some recognizable fragments are indicated in the legend to fig.3.

The identical chromatographic behaviour, presence of sulfur, identical M^+ and identical fragmentation support the identity of endogenous TMDA with the synthetic compound. A rough quantitative evaluation of TMDA contained in the brain gives a value of 1 μg per cent g brain. This value, however, is largely indicative because great variations have been observed between different extracts. Accurate quantitation of TMDA and cyclothionine must await a more sensitive and simpler method of analysis, which we are at present working on.

4. DISCUSSION

After cyclothionine [7], TMDA is the second S-containing cyclic imino acid so far discovered in brain. While the biosynthesis of cyclothionine is easily amenable to an enzymatic conversion of cystathionine, which is present in large amounts in mammalian brains [11], the biosynthesis of TMDA is much more difficult to understand. Apart from its possible external source, the monodeamination of lanthionine followed by its cyclization and reduction, as outlined in fig.1, could be a plausible mechanism. Enzymatic deamination and cyclization of lanthionine have been reported [8]. Lanthionine, however, is a rare amino acid found as such only occasionally in biological material [12–14], being known more as a product of denaturation of cystine-containing proteins [15] than as a metabolic compound. The enzymatic production of lanthionine was described, in Braunstein's laboratory in 1969, through the action of serine sulfhydrase [16] but has never been studied in detail since then. Apart from the derivation from lanthionine, other paths could be followed for the production of TMDA. For instance the β-replacement of serine or cysteine by some unspecific lyase [17] using mercaptopyruvate as substituent could produce lanthionine ketimine which when reduced yields TMDA.

A point of relevance which stems from the present and previous work [7] is the occurrence in brain of acidic N-S containing 7- and 6-membered rings suggestive of a possible functional role for these compounds totally ignored at present.

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