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Two new reactive targets of 2,5-hexanedione in vitro – beta-alanine and glycine

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Summary. In this study, we found that two amino acids reacted with 2,5-hexanedione to form new reaction products in vitro, respectively. In the reaction of beta-alanine and 2,5-hexanedione, a reaction product was obtained and analyses of obtained results showed it was 3-(2,5-dimethyl-1*H*-pyrrol-1-yl)propanoic acid; in the reaction of glycine and 2,5-hexanedione, a reaction product was also obtained and analyses showed it was (2,5-dimethyl-1*H*-pyrrol-1-yl)acetic acid. Two reaction products were found to be oxidized easily; in addition, the latter was more easily to be oxidized than the former in the air. Our discoveries demonstrated that reactions between amino acids and 2,5-hexanedione could exist possibly in vitro. At present, it is clear that 2,5-hexanedione causes either axon atrophy or swelling, but the underlying molecular mechanism is still unclear. Since both beta-alanine and glycine are considered as neurotransmitter in the central nervous system, the reaction products remain to be identified in vivo.

Keywords: Gamma-diketone neuropathy – 2,5-Hexanedione – Beta-alanine – Glycine – Neurotransmitter – Amino acids

Introduction

The neuropathy due to *n*-hexane was initially discovered in humans. Previous research has discovered that repeated exposure to *n*-hexane or methyl *n*-butyl ketone leads to neuropathy in human (Spencer et al., 1980; Allen et al., 1975; Yamamura, 1969). In addition, metabolism studies have demonstrated that the neuropathy induced by these hexacarbons is mediated by the common metabolite, 2,5-hexanedione (2,5-HD) (DeCaprio et al., 1983; Zhu et al., 1994; Misumi et al., 1997; Zhang et al., 2005).

Beta-alanine, a constituent of coenzyme A, is an unusual amino acid that is absent from protein (Bonfanti et al., 1999). Like glycine, it exhibits neurotransmitter activity and is a component of the anti-glycation agent carnosine (Mehta and Seidler, 2005). Beta-alanine is also the carboxylic acid analogue of taurine, 2-aminoethanesulphonic acid, a probable inhibitory neuromodulator in the central nervous system (CNS) (Kontro, 1983). However, the concentration of beta-alanine in the CNS is low, 0.02–0.10 mmol/kg with a large heterogeneous regional distribution (Kontro, 1983; Perry et al., 1971). In addition, beta-alanine has also been proposed to be involved in synaptic transmission (Kontro, 1983; DeFeudis et al., 1977).

On the other hand, glycine is a non-essential amino acid synthesized by animals. Of all the 20 amino acids commonly found in animal protein, glycine is the simplest and the only one that is not optically active. It is present in most tissues and a key substance in the metabolism of one-carbon fragments, proteins, peptides, nucleotides, porphyrins, and bile salts. It is also a major inhibitory neurotransmitter in the spinal cord and brain stem, and an anti-inflammatory, cytoprotective, and immune modulating substance (Gundersen et al., 2005).

Furthermore, both beta-alanine and glycine exist in many tissues, such as aorta, ventricle, atria, liver, kidney, pancreas, bronchi and adrenals (Decker et al., 1995).

Although the mechanism of gamma-diketone neuropathy remains unknown, pyrrole-forming reactions are considered to be the first step. It was hypothesized that 2,5-hexanione might bind with protein lysine epsilon-amine moieties to yield 2,5-dimethylpyrrole adducts, and pyrrolyzation represented the critical initiating event in gamma-diketone-induced neuropathy (DeCaprio et al., 1988; Graham et al., 1982; DeCaprio and O'Neill, 1985;

Anthony et al., 1983; DeCaprio and Fowke, 1992). Concerning the reactivity of 2,5-hexanedione, Graham et al. (1982) also demonstrated that 2,5-hexanedione reacts with ethanolamine to form 1-(2-hydroxyethyl)-2,5-dimethylpyrrole. And sequentially the pyrrole was found to autoxidize to form an orange chromophore. Whereas, at present, the reaction relationship between 2,5-hexanedione and certain neurotransmitters under approximate physiological condition still remains unclear, especially the free amino acids.

Therefore, the aim of this study is to explore the reactivity of 2,5-hexanedione with certain neurotransmitters, and to shed more light into our understanding of the various properties of certain amino acids through in vitro study.

Materials and methods

2,5-hexanedione ($CH_3COCH_2CH_2COCH_3$, 97% pure), beta-alanine ($NH_2CH_2CH_2COOH$, 97% pure) and glycine (NH_2CH_2COOH, 99% pure) were obtained from Wako Pure Chemicals Co. (Osaka, Japan). All other chemicals were of reagent grade.

1.25 mmole of beta-alanine was dissolved in 1 ml of 0.9% saline with control tubes, and 5 mmole of 2,5-hexanedione was added to the resulting solution. Incubation was conducted at 37 °C for more than 48 hours. The solution became muddy afterwards, and black oil chromophores could be found at the bottom of test tube, while the control tubes remained unaffected. Glycine was also incubated with 2,5-hexanedione base on the procedures described above.

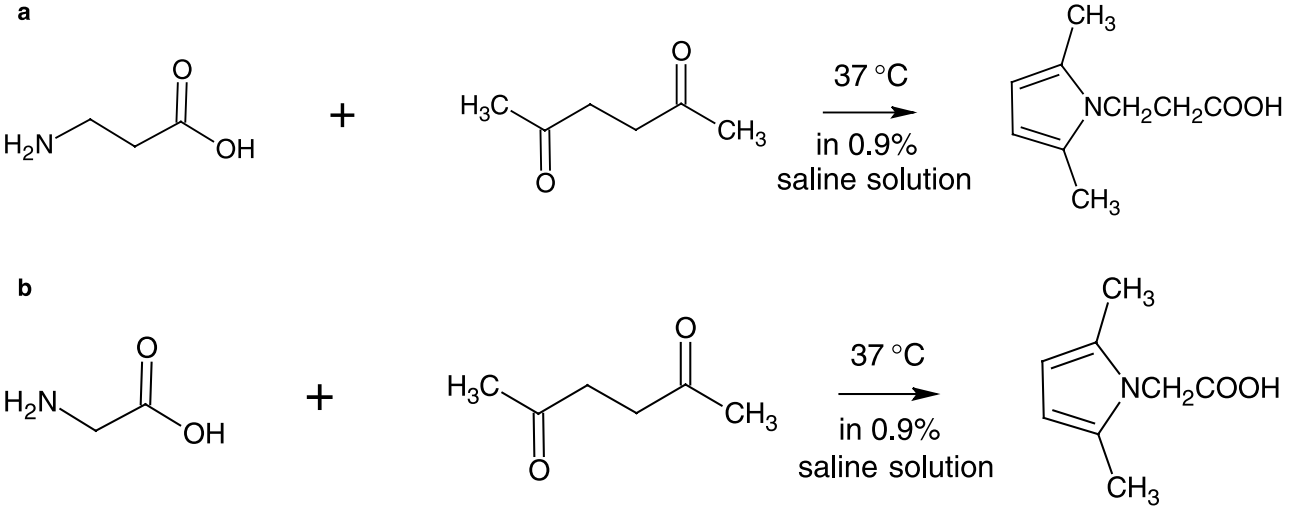
Then the solution obtained was mixed with 10 ml of chloroform and agitated for 5 minutes. This procedure was repeated three times. The chloroform layers were collected and evaporated under a stream of nitrogen. The extracts were resuspended in chloroform and were injected into a Shimadzu GCMS-QP2010 GC/MS system using Rtx-200 column. After the column chromatography was performed, the fractions were developed with chloroform, methanol and acetic acid (80:20:a drop) to recover the pure reaction products for ensuing thin-layer chromatography. Then the fraction of reaction product was streaked on silica gel plates and developed with chloroform, methanol and acetic acid (80:20:a drop) after being concentrated under a stream of nitrogen. The reaction product was extracted with chloroform and chloroform was subsequently evaporated.

Finally, proton nuclear magnetic resonance ^{1}H-NMR spectra were recorded with a Bruker ARX-300 ^{1}H-NMR spectrometer. The experiment of oxidability of the reaction product was performed by the spectrophotometric method using a Jasco V-530 spectrophotometer. The identification of the reaction product from the reaction of glycine and 2,5-hexanedione was also conducted as described above.

Results

Both ^{1}H-NMR spectrum and mass spectrum of the reaction product formed from the reaction of beta-alanine with 2,5-hexanedione were recorded. The data were shown as follows: ^{1}H-NMR (300 MHz, $CDCl_3$) $\delta = 2.23$ (6H, s, 2-CH_3 and 5-CH_3), 2.65 (2H, t, $J = 7.8$ Hz, –CH_2–CO–), 4.06 (2H, t, $J = 7.8$ Hz, N–CH_2–), 5.77 (2H, s, 3-H and 4-H), 10.06 (1H, s, –COOH). MS: m/z (%) = 167 (M^+, 100), 152 (13), 122 (17), 108 (50), 94 (61). These data showed that this reaction product was 3-(2,5-dimethyl-1*H*-pyrrol-1-yl)propanoic acid, MW = 167; its reaction process is shown in Scheme 1a. Furthermore, its mp value was determined at 70–71 °C. The absorption spectrum of 3-(2,5-dimethyl-1*H*-pyrrol-1-yl)propanoic acid in the aqueous solution of KOH disclosed that this compound was easily oxidized in the air, and showed a progressive formation of an orange chromophore with λ_{max} between 380 and 480 nm after 18 h of purification, as shown in Fig. 1.

On the other hand, the same experiments were conducted to identify the reaction product formed from the reaction of glycine and 2,5-hexanedione. The data were shown as follows: ^{1}H-NMR (300 MHz, $CDCl_3$) $\delta = 2.17$ (6H, s,



Scheme 1. Pyrrole synthesis formed from the reaction between beta-alanine and 2,5-hexanedione (**a**); and the reaction between glycine and 2,5-hexanedione (**b**), respectively

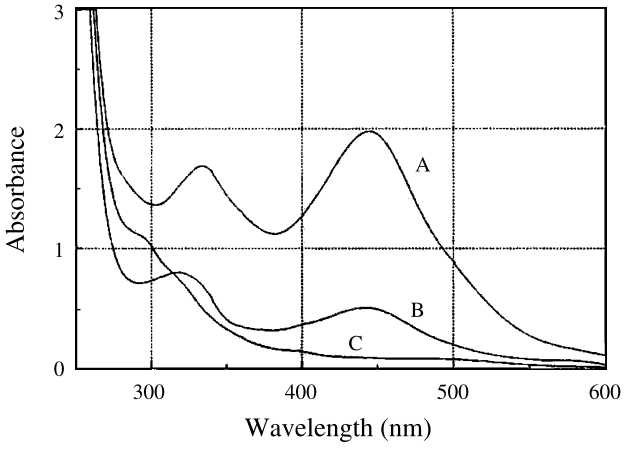


Fig. 1. Absorption spectrum of 3-(2,5-dimethyl-1*H*-pyrrol-1-yl)propanoic acid formed in the reaction of beta-alanine with 2,5-hexanedione. The colourless crystals were dissolved in the aqueous solution of KOH (0.5 mg/ml), following purification through thin-layer chromatography. *A*, *B*, and *C* represent an absorption spectrum after 18 h of purification; an absorption spectrum after purification; an absorption spectrum in which oxidized pyrrole of 3-(2,5-dimethyl-1*H*-pyrrol-1-yl)propanoic acid has been reduced by $NaBH_4$ after 18 h of purification, respectively

2-CH_3 and 5-CH_3), 4.54 (2H, s, –CH_2–), 5.83 (2H, s, 3-H and 4-H), 9.46 (1H, s, –COOH). MS: m/z (%) = 153 (M^+, 100), 138 (12), 108 (83), 94 (55). The reaction product was (2,5-dimethyl-1*H*-pyrrol-1-yl)acetic acid, MW = 153; its reaction process is shown in Scheme 1b. However, in TIC of reaction products of glycine and 2,5-hexanedione, there were two novel peaks at 17.2 min; (MW = 173) and at 19.0 min; (MW = 187) (data not shown). Unfortunately,

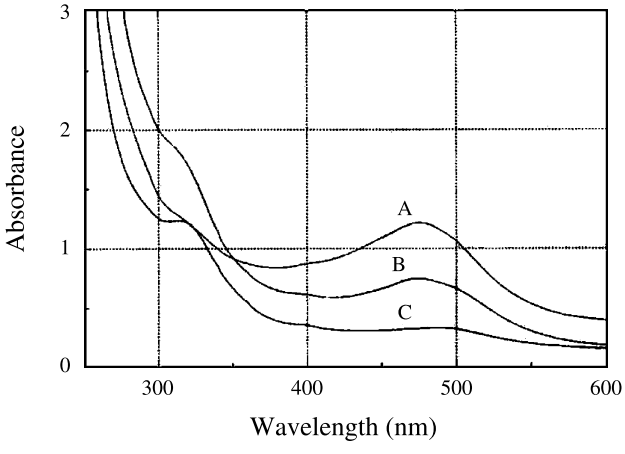


Fig. 2. Absorption spectrum of (2,5-dimethyl-1*H*-pyrrol-1-yl)acetic acid formed in the reaction of glycine with 2,5-hexanedione. The colourless crystals were dissolved in the aqueous solution of KOH (0.5 mg/ml), following purification through thin-layer chromatography. *A*, *B*, and *C* represent an absorption spectrum after 18 h of purification; an absorption spectrum after purification; an absorption spectrum in which oxidized pyrrole of (2,5-dimethyl-1*H*-pyrrol-1-yl)acetic acid has been reduced by $NaBH_4$ after 18 h of purification, respectively

the structures of these reaction products remain unknown. Due to its oxidation property, we were unable to determine the mp value of (2,5-dimethyl-1*H*-pyrrol-1-yl)acetic acid under the present condition. Moreover, the absorption spectrum of this compound in the aqueous solution of KOH indicated that this compound was also easily oxidized in the air, as shown in Fig. 2.

Furthermore, we found that (2,5-dimethyl-1*H*-pyrrol-1-yl)acetic acid was more easily oxidized than 3-(2,5-dimethyl-1*H*-pyrrol-1-yl)propanoic acid in the air through our experiments.

Discussion

Despite that *n*-hexane is an organic solvent widely used in the preparation of fabrics, adhesives, lacquers and other coatings for many years, we only possess very few knowledge concerning its property, especially that of its common metabolite, 2,5-hexanedione.

Exposure of humans and experimental animals to the hexacarbons, *n*-hexane or methyl *n*-butyl ketone induces nerve damage characterized as a central-peripheral distal axonopathy. Recently, it is widely considered that 2,5-hexanedione causes either axon atrophy or swelling base on the various daily doses to the experiment animals, but the underlying molecular mechanism is still unclear. Although a number of researches have been conducted to discern the mechanism of neuropathy over the past 25 years, only a few of them have investigated the neurotoxicity of 2,5-hexanedione, specifically that of reactivity of 2,5-hexanedione with certain amino acids and amino acid residues. Thus, we consider that our research to identify definite reactivity of 2,5-hexanedione with certain amino acids has provided valuable addition to the literature.

In our prior studies, we had failed to demonstrate that four amino acids possessing free amino groups, namely arginine, glutamine, lysine, and asparagine, reacted with 2,5-hexanedione within rat axon. However, our latest discovery that both beta-alanine and glycine react with 2,5-hexanedione to form two novel reaction products suggests that the amino groups of these two amino acids are active targets for the attacks of 2,5-hexanedione, and the ensuing pyrrole formation could be considered as an inevitable result to explain the action of 2,5-hexanedione to amino groups of certain amino acids. Our current results not only confirm reports of Graham et al. (1982), which presented the evidence for pyrrole formation of lysyl residues in vitro, but also demonstrate the reactivity of 2,5-hexanedione to amino groups of certain amino acids under approximate physiological condition. Furthermore, our results suggest

that the autoxidation of reaction product led to the orange chromophore generated.

Since the above two amino acids play an important role in CNS, for example, beta-alanine exhibits neurotransmitter activity in CNS; and glycine is also a major inhibitory neurotransmitter in the spinal cord and brain stem, the next step is to identify these two reaction products in vivo tissues. Our attempts to extract them in vivo by chloroform have been unsuccessful, which might be caused by the autoxidation of pyrroles and relatively lower contents of the two amino acids in CNS. These two factors might directly lead to multiple monomeric and polymeric products to be generated, relatively lower recovery yields, and difficulty of directly identifications using current method.

At present, although our in vitro study provides new evidences for predicting potential neurotoxicity of 2,5-hexanedione in inducing neuropathy, the two new reaction products we have found still remain to be identified in vivo by novel method. Since if the identifications of them in vivo are possible, this could provide a new approach for discerning the molecular mechanism of gamma-diketone neuropathy. In addition, the elucidation of differences in the contents of amino acids in vivo tissues might also be a focus for identifying these two novel reaction products. We hope that we can successfully develop a new method of extracting these novel reaction products in vivo tissues in the near future.

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