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The Mechanism of the Biochemical Activity of 3,5-Diphenylamine-1,4 Dihydro-2,6-Dimethylpyridine Dicarboxylate Adduct of Formaldehyde and Plasma Albumin Cross Link

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Abstract

Formaldehyde and proteins cross link adducts have variously been reported and also associated with some health risk factors like diabetes. In this study, an 3,5-diphenylamine-1,4 dihydro-2,6-dimethylpyridine dicarboxylate adduct was prepared from a mixture of Formaldehyde, Acetoacetinalide and Plasma Albumin and was characterized using melting point, IR, Uv spectra characteristics as well as the antimicrobial activity based on the method described by Hantzsch. The adduct was found to have a melting temperature range of (179.5 - 180.4°C). The result of the FTIR analysis showed the functional groups present in the adduct as follows: The band at 2948.85 cm^{-1} was assigned to aldehyde (HC=O) group. The ones at 3850.5 cm^{-1} 3736.73 and 3617.47 were found to be (O-H) stretch for water of hydration and N-H stretches. The sharp 2356.87 band was found to be due to N-H stretch from amines in the adduct while the bands at 1739.68, 1547.01 cm^{-1} and 1032.63 cm^{-1} were attributed to C=O and C-H stretch as well as C-N stretch due to tertiary amines respectively. The Antimicrobial screening test on the adduct also showed some antimicrobial effect to some micro-organism such *as Bacillus sustilis (12.0 mm), Proteum mirabilis (16.0 mm) and Canidad albicans (14.0 mm)* as their growth was inhibited when placed around their growth media. It was however found to have no effect on some fungi and algae such as *Escherichia coli and Staphylococcus aureus*. The antimicrobial activity properties have been found to be due to the aldehyde group and the mannan -like binding properties from the adduct acting as a glycoprotein.

1. Introduction

Formaldehyde and protein cross links reactions have variously been reported and have

been associated with some health risk factors [1]. Similarly in vivo non-enzymatic reaction between reducing sugars and proteins in glacylation reactions (the covalent bonding of blood glucose to the red blood cells) have been reported for formaldehyde. Various methods have been proposed by various scientists for the synthesis of formaldehyde and protein adducts like 1,4-dihydropyridine. Synthesis of 1,4-dihydropyridines was first reported by Hantzsch by refluxing of aldehyde, β-ketoester and ammonia or ammonium salts in ethanol and has been found to form various substituted dihydro-2,6-dimethylpyridine [3]

Glycation, a chemical modification of proteins with formaldehyde or reducing sugars, indicates a possible explanation for the association between hyperglycemia and the wide variety of tissue pathologies [4, 5]. Various Researches have suggested that formaldehyde and reducing sugars can react with the amino groups of long-lived proteins to produce non-enzymatic cross-links [6, 7]. Formations of these cross-links occur as end-stage products of the Maillard reaction; they are known as advanced glycation end-products (AGEs) [4]. AGEs are a class of complex, often unstable, reactive compounds formed in excess during aging and diabetes mellitus [5]. Some of the protein alterations observed in diabetic patients resemble those in much older, non-diabetic patients, suggesting diabetes induced early aging [8]. Protein glycation and AGE formation are accompanied by increased free radical activity that contributes to the bimolecular damage in diabetes [6]. AGEs act as mediators and can initiate a wide range of abnormal responses in cells and tissues such as the inappropriate expression of growth factors, alterations in growth dynamics, accumulation of extra-cellular matrix and initiation of cell death through decreased solubility, elasticity and enzymatic affinities in long-living proteins such as collagen [4].

A number of these chemical and physical skin changes are reported in human skin collagen with age and appear to be accelerated in diabetes [9]. AGE cross-linking reactions in collagen contributes to diabetic circulatory complications such as vascular stiffening and myocardial dysfunction [10, 5]. Although the mechanisms underlying the development of the complications of diabetes are not fully understood, there is now a consensus that hyperglycemia does play an important role in the development of retinopathy, nephropathy, neuropathy and joint stiffness. For example, increased serum and tissue levels of AGEs due to a reduced removal by the kidneys have been evident in end-stage renal failure. *In vitro* and *in vivo* studies have shown that AGEs result in irreversible cross-links in long living matrix structural proteins such as type IV collagen, laminin and fibronectin [5].

A major consequence of hyperglycemia is excessive non - enzymatic glycosylation of proteins, primarily due to long-term exposure to elevated glucose concentrations [10]. Non - enzymatic glycation may be occurring, although at a much slower rate than that seen most diabetic mellitus (DM) patients [9]. Non enzymatic protein glycation (Mallard Reaction) by glucose is reported to be a complex cascade of condensations, rearrangements, fragmentations and oxidative modifications. Glucose is found to chemically attach to proteins and nucleic acids without the aid of enzymes, increasing the formation of AGEs. These AGEs form on intra- and extracellular proteins, lipids, and nucleic acids, leading to the generation of protein fluorescene and the irreversible cross-links [11]. The formation of AGEs requires the reaction of reducing sugars like glucose, fructose, galactose, mannose and ribose. Interestingly, glucose is among the least reactive of the common sugars, perhaps leading to its evolutionary selection as the principle free sugar *in vivo* [10].

For a given protein, the extent of non enzymatic glycosylation is determined by the sum of effects of a number of independently acting variables such as pH, temperature, protein concentration etc [11, 12]. Glucose concentration and incubation time are the most clinically relevant variables affecting the extent of non-enzymatic glycosylation. Characteristic to diabetics, increased levels of glucose concentrations cause the level of accumulated Amadori products on proteins to rise [11, 9]. Non-enzymatic glycosylaton is a common posttranslational modification of proteins *in vivo*, resulting from reactions between glucose and amino groups on proteins, this process is coined the "Maillard reaction" and results in the formation of AGEs [11].

This study therefore investigated the mechanism of the biochemical activity of the cross link adduct of the reaction between formaldehyde and plasma albumin in ethanol – water binary mixtures with the view to understanding and shading more light with the view to relating it as risk factor for diabetes and other related human complications.

2. Method

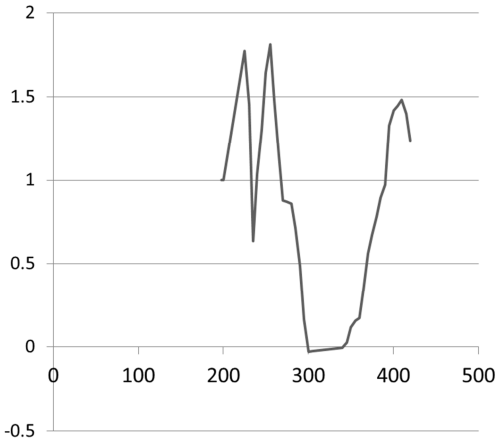

Figure 1. *The three wavelengths of maximum absorptions λ_{max} of the 1,4-dihydrolutidine adduct at 250, 255 and 410 nm.*

The Plasma albumin used in this study was isolated, purified and fractionated using ethanol based on the methods of [11, 12]. The 3, 5-Diphenylamine-1, 4 dihydro-2,6-dimethylpyridine dicarboxylate adduct variously reported

was prepared from a mixture of Formaldehyde, Acetoacetinalide and Plasma Albumin and was characterized using melting point, IR, Uv spectra characteristics as well as the microbial activity based on the method described by Hantzsch as presented in Figures 1, 2 and 3 [3, 2] as follows: A mixture of 20 cm^3 of acetoacetanilide (0.02 mol dm^{-3}), 20 cm^3 of (0.0001 mol dm^{-3}) formaldehyde and 10cm^3 of (0.0001 mol dm^{-3}) plasma albumin were taken in a reflux flask and refluxed at a temperature of 25.0°C for one hour on a water bath. The contents were transferred in a beaker and left to stand overnight after which it was filtered to obtain the yellow dihydrolutidine derivative. The adduct was recrystallized from water and dried over potassium permanganate in a desiccator. The prepared adduct was analysed for its melting point, maximum absorption using uv/visible spectrophotometer and its functional groups using Fourier transform infrared spectroscopy as well as its antimicrobial susceptibility test [3].

Peak finding results for: amino acid

Frequency: 406.75 - 4000.00, threshold: 88.086, sensitivity: 50.00

Peak finding result table:

Peak#	1	2	3	4	5	6	7	8	
Position	3850.70	3736.73	3617.47	2357.04	1739.68	1547.01	1032.33	670.73	
Height	69.126	67.360	68.292	52.241	74.880	75.780	78.714	83.211	

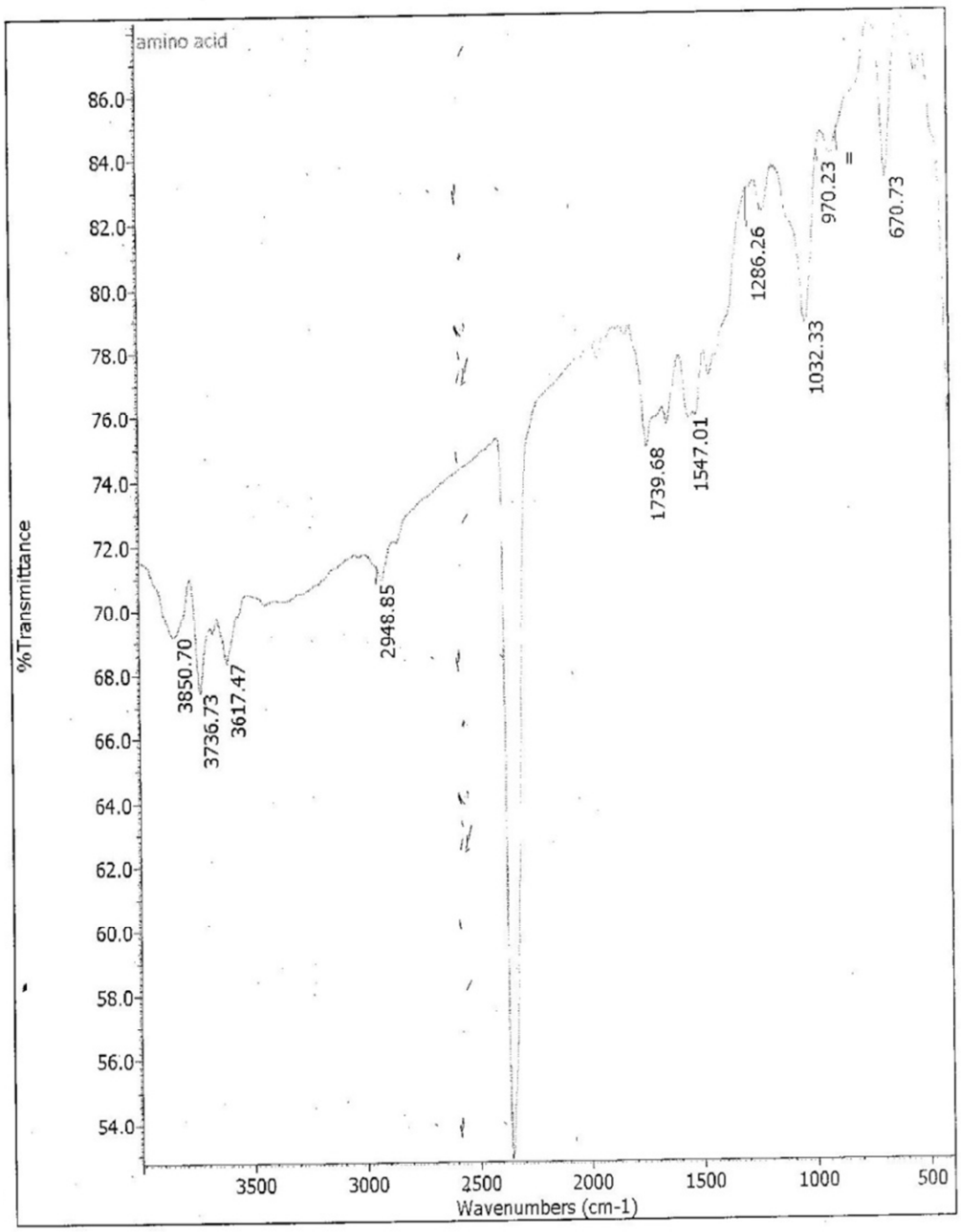

Figure 2. *The Percentage Transmittance Bands and Intensities in Wave Numbers (cm^{-1}) of Plasma Albumin – Formaldehyde Adduct (Ugye et al, 2011).*

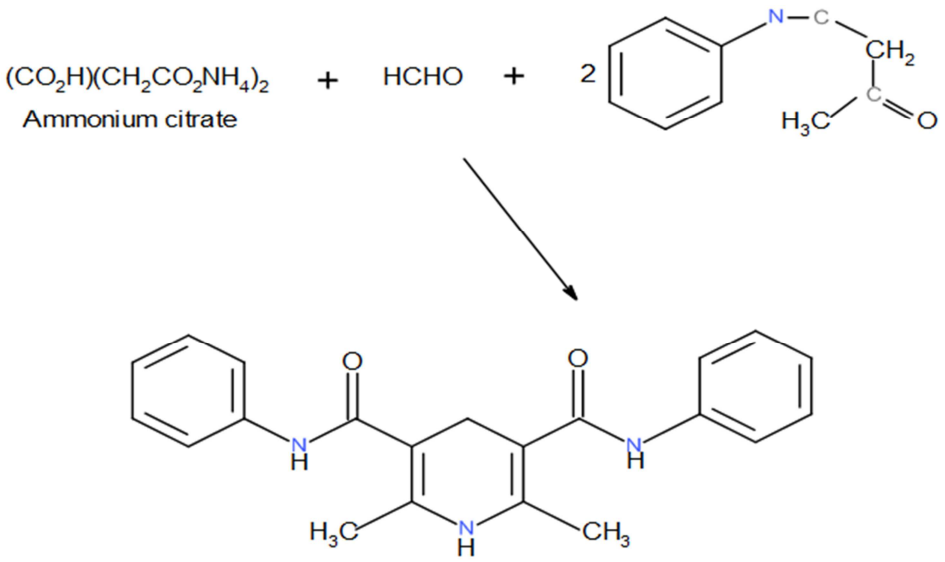

Figure 3. *Reaction scheme of acetoacetinalide and formaldehyde in the presence of ammonia derived from ammonium citrate to form The 3,5-diacetyl -1, 4 dihydropyridine dicarboxylate (Li et al., 2007; Ugye et al., 2011) [1].*

3. Results and Discussion

The synthesised adduct was found to absorbed the ultra violet light strongly at three wavelengths (λ_{max}) 250, 255 and 410 nm with an absorption coefficients (ϵ) of 1645, 1812 and 1483L mol^{-1} cm^{-1} respectively as presented in Figure 1.

The result of the melting point analysis as given in Table 1 showed that the adduct had a melting point range of (179.8 - 180.4°C). This indicated that the adduct is labile Schiff base with similar characteristics with those found in the literature which were referred to gylcated products [14]. The result of antimicrobial activity analysis which is presented in Table 2 showed that the 1,4-dihydropyridine adduct has some antimicrobial effect on some micro-organisms such *as Bacillus subtilis* zone of inhibition 12.0 mm, *Proteum mirabilis* zone of inhibition, 16.0 mm *and Canidad albicans* zone of inhibition, 14.0 mm The adduct have no effect on some micro organisms such fungi /algae like *Escherichia coli* and *Staphylococcus aureus*. This shows that the adduct has no active antimicrobial activity over fungi and algae. This property was reported to be typical of lectin action mechanisms, and it was observed that the 1.4-dihydropyridine adduct had the specialized plyco-protein properties [15].

These findings from the study have therefore shown that the prepared adduct is a glycoprotein with an among other functional groups, an aldehyde lectin-like moiety and is a Schiff base just as the in vivo non-enzymatic reaction between reducing sugars and proteins in glacylation (the covalent bonding of blood glucose to the red blood cells) through hemiacetyl formation mechanism.

Human blood proteins like hemoglobin and serum albumin are elsewhere reported to undergo slow non - enzymatic glycation with oxidizing agents such as formaldehyde, mainly by forming schiff bases between ϵ- amino groups of lysine and sometimes arginine and glucose molecules in the blood [16]. Also elevated glycol albumin has been reported in diabetes mellitus [17] and biological activity of various 1, 4 dihydropyridines derivatives have been published in various papers [18, 19]. More so the chemical nature of AGEs *in vivo* is largely unknown, but there are growing population of structurally-defined AGE adducts such as pyrraline, pentosidine, N-carboxy-methyl lysine (CML) and crosslinks that are found to be elevated in diabetic tissues [19]. The best found chemically characterized AGEs in humans are pentosidine and CML (Figures 4, and 5). Some of the highest levels of pentosidine have been detected in individuals afflicted with Diabetes Mellitus [19]. Evidence has shown that elevated skin pentosidine levels in individuals with diabetes mellitus correlate with the severity of the complications. Initial investigations have shown that pentosidine can be detected in smaller levels in various tissues of non-collagenous origin, including the blood and the human lens. Pentosidine is a fluorescent crosslink with visible wave length fluorescence, making it easy to detect. Methods for synthesizing and detecting AGEs such as pentosidine have been proposed in various studies [19].

The pathogenesis of diabetic complications continues to be a central issue in current diabetes research. Diabetes mellitus is one of the most prevalent metabolic syndromes world-wide, and is characterized by hyperglycemia resulting in short-term metabolic changes in lipid and protein metabolism and long-term irreversible vascular and connective-tissue changes. These changes include diabetes-specific complications such as retinopathy, nephropathy and neuropathy and complications of the macro-vasculature such as atherosclerosis; potentially resulting in heart disease, stroke and peripheral vascular disease [19]. Links between chronic hyperglycemia and the development of long-term diabetic-specific complications have been discovered and are yet not completely understood [6].

Table 1. *Melting Point Range of Plasma Albumin – Formaldehyde Adduct.*

Initial melting point °C	Final melting point °C
179.5	180.2
180.0	180.4
180.0	180.5
Mean sample melting point range	179.8 -180.4

Table 2. *Antimicrobial Activity of Plasma Albumin –Formaldehyde Adduct.*

Test organisms	Zone of inhibition (mm)
Bacillus sustilis	12.0
Proteum mirabilis	16.0
Canidad albicans	14.0
Escherichia coli	0.00
Staphylococcus aureus	0.00

Primary amino groups ($-NH_2$) such as N-terminal and lysine α- amino groups of proteins, are known to react rapidly with the simple members of aldehyde group to form Schiff bases $–N=CHCH_3$. The Schiff bases are however found to be unable to react further with alcohols [21]. These workers however reported that like some previous work, they found the exo cyclic amino groups of nucleic acid components react quickly at ambient temperature with acetaldehyde and ethanol to yield mixed acetals [$R-NH-CH(CH_3)-O-C_2H_5$]. Their work also found that the same type of reaction occurs readily with the nitrogen of 3-substituted indoles (e.g indole-3-acetic acid and N-acetyltryptophan), analogues of the amino acid tryptophan.

This study group also found previously that the rate of the reaction of formaldehyde with plasma albumin in ethanol-water mixtures preceded from formation of hemiacetyl groups to acetals and decreased with increase in the percentage ethanol – water mixtures [1]. Thus the mechanism of the biochemical activity of the prepared adduct and formaldehyde in particular in the human body may be said to be by covalent binding by the aldehyde group to sugar moieties in cell walls or membranes and thereby changing the physiology of the membrane to cause agglutination, mitosis, or other biochemical changes in the cell [21]. The adduct may also undertake the non-enzymatic Glycation which might lead to human health complications and diseases such as diabetes [23, 24].

4. Conclusion

(a) The findings from this study have shown that the 3, 5-Diphenylamine-1,4dihydro-2,6-dimethylpyridine dicarboxylate an adduct obtained from formaldehyde and plasma albumin cross link is a glycoprotein with an aldehyde moiety

(b) That the adduct exerts the observed antimicrobial activity through covalent binding by the aldehyde group to sugar moieties in cell walls or membranes just as the reported in vivo non-enzymatic reaction between reducing sugars and proteins in glacylation (the covalent bonding of blood glucose to the red blood cells).

(c) The adduct is capable of specific recognition as well as reversible binding to carbohydrate moieties of complex glycol conjugates and hemiacetals without altering the covalent structure of any of the recognized glycosyl ligands and could be associated with diabetes

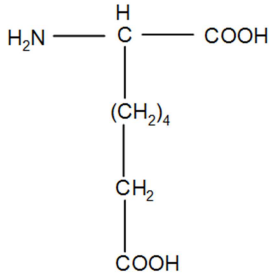

Figure 4. *Structure formed by glycation of lysine residues in protein, fructoselysine (FL) (Wautier, and Guillausseau, 2001).*

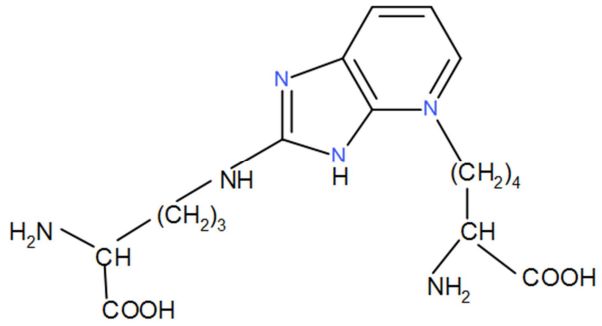

Figure 5. *Structure of fluorophore P (Pentosidine). (Wautier, and Guillausseau, 2001).*

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