[C0031]

Novel forms of tryptophan glycoconjugates: chemical *versus* enzymatic glycosylation

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INTRODUCTION

Until recently, investigations on the glycosylation of amino acids during the Maillard reaction, on the formation of advanced glycation endproducts (AGE) and the postranslational modification of proteins mainly concentrated on the amino acids lysine, arginine, asparagine, serine and threonine whereas the significance of tryptophan remained largely unnoticed. While studying occurrence and relevance of novel tryptophan metabolites in biological systems, our attention on tryptophan glycosylation was attracted by reports on mannosylated tryptophan residues in proteins [1-6]. In this contribution, we describe the characterization and structure elucidation of novel tryptophan glycoconjugates resulting from the chemical condensation of tryptophan and aldohexoses and demonstrate their occurrence in food samples [7]. In addition, we report on the identification of tryptophan-*N*- and -*C*-glycoconjugates, namely N^1-(b-D-glucopyranosyl-$^4C_1$)-L-tryptophan and 2-(a -manno-pyranosyl-$^1C_4$)-L-tryptophan, formed enzymatically as novel tryptophan metabolites in plants [8] and man [7].

CHEMICAL FORMATION OF TRYPTOPHAN GLYCOCONJUGATES

With regard to the reactivity of the indole moiety towards electrophilic attack, several reaction products of tryptophan with aldohexoses could be postulated (Figure 1).
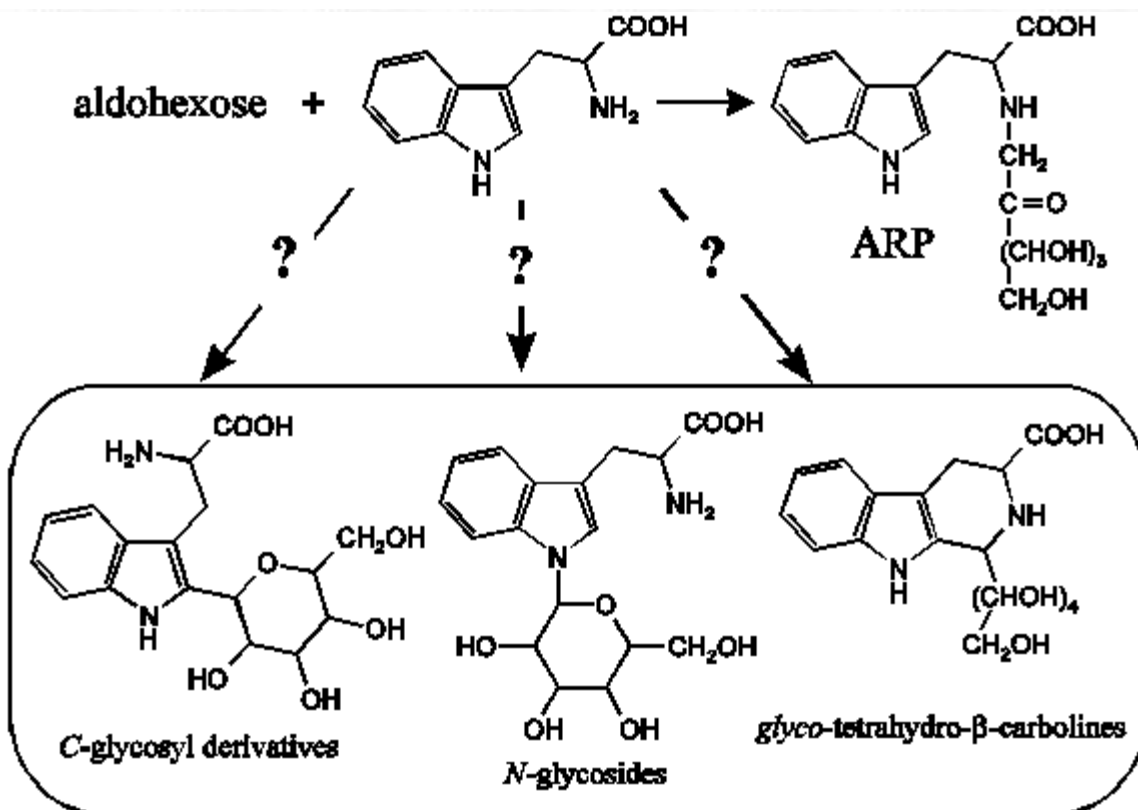
Figure 1. Postulated reaction products of tryptophan with aldohexoses.

Besides the well-known Amadori rearrangement product (ARP), we considered tryptophan-*N*-glycosides, -*C*-glycosyl derivatives and *glyco*-tetrahydro-b-carbolines as primary condensation products. To prove formation of these novel tryptophan glycoconjugates, we performed model reactions heating tryptophan with D-glucose, D-galactose and D-mannose to 80 °C at pH 2 for 12 days. HPLC-ESI-MS analysis of each model reaction yielded up to four tryptophan glycoconjugates from each of the aldohexoses with molecular ions m/z 367 [M+H]$^+$ other than the established Amadori rearrangement product [9]. Subsequent isolation of the compounds by RP-18 column chromatography and characterization by tandem mass spectrometry combined with NMR spectroscopy including HH-COSY, HMBC and HMQC experiments led to identification of the postulated structures [7].

The product ion spectrum of the tryptophan-*N*-glycosides as obtained by low-energy CID of the molecular ion m/z 367 [M+H]$^+$ is dominated by ions m/z 205 and m/z 188 corresponding to the loss of an intact anhydro-sugar moiety $C_6H_{10}O_5$ and subsequent loss of NH_3 (Figure 2A). This fragmentation pattern is characteristic for *N*-glycosidically linked sugars [10] whereas in product ion spectra of *C*-glycosyl derivatives and *glyco*-tetrahydro-b-carbolines the prominent ions m/z 247 and m/z 229 result from loss of a $C_4H_8O_4$ moiety and subsequent loss of H_2O (Figure 2B).

A

product ion (m/z)	fragmentation
350	[M+H-NH$_3$]
332	[350-H$_2$O]
247	[M+H-C$_5$H$_8$O$_4$]
229	[247-H$_2$O]
205	[tryptophan+H]
188	[205-NH$_3$]

B

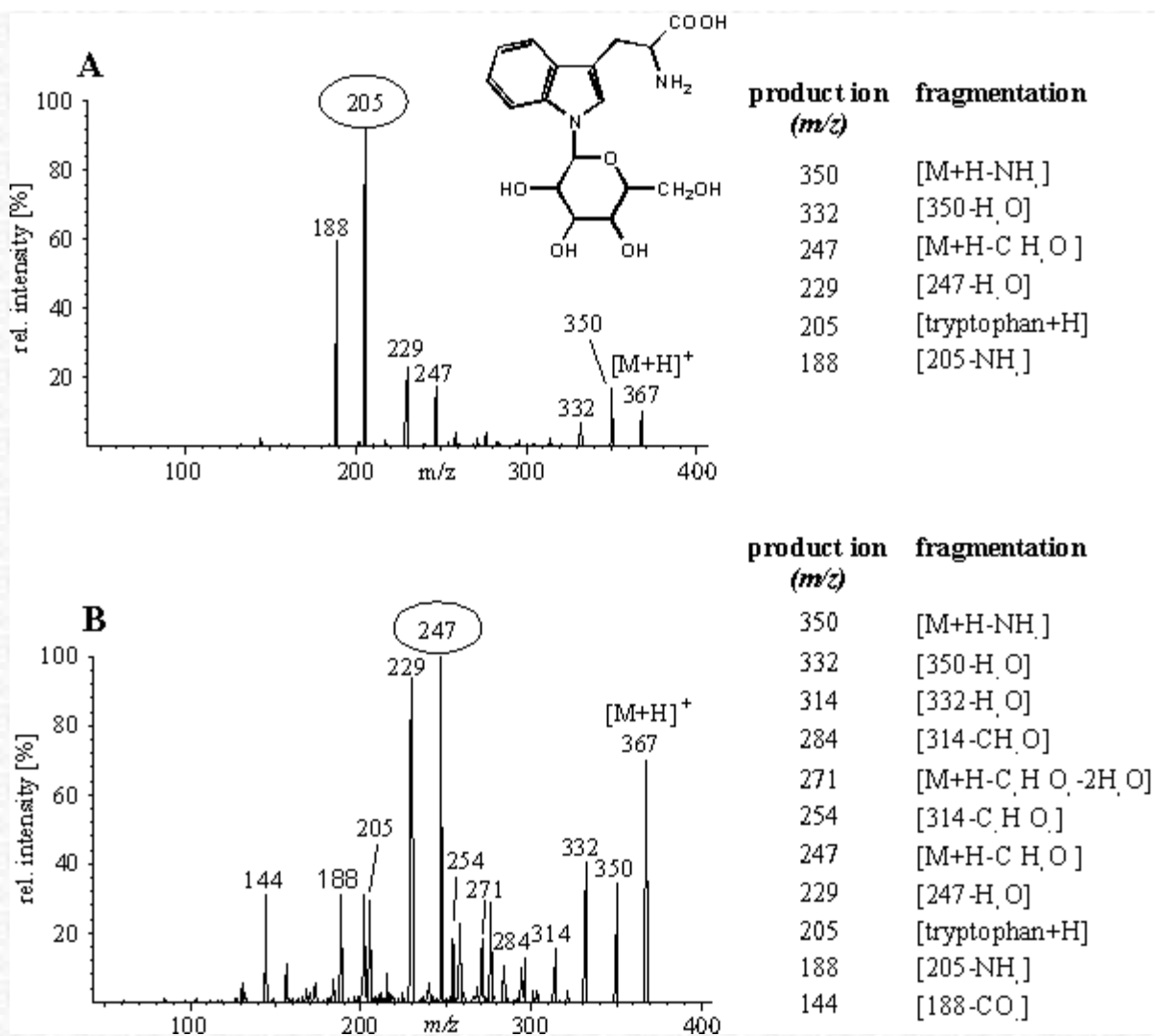| product ion (m/z)	fragmentation
350	[M+H-NH$_3$]
332	[350-H$_2$O]
314	[332-H$_2$O]
284	[314-CH$_2$O]
271	[M+H-C$_2$H$_4$O$_2$-2H$_2$O]
254	[314-C$_2$H$_4$O$_2$]
247	[M+H-C$_5$H$_8$O$_4$]
229	[247-H$_2$O]
205	[tryptophan+H]
188	[205-NH$_3$]
144	[188-CO$_2$]

Figure 2. Product ion spectra of tryptophan glycoconjugates, precursor ions *m/z* 367 [M+H]$^+$ (positive mode ESI, 19 eV; 267 mPa Ar); A: tryptophan-*N*-glucoside; B: tryptophan-*C*-glucosyl conjugate and *gluco*-tetrahydro-β-carbolines.

Differentiation between *C*-glycosyl derivatives and *glyco*-tetrahydro-b-carbolines was achieved by low energy CID of the deprotonated molecular ions *m/z* 365 [M-H]$^-$. Product ion spectra of *C*-glycosyl derivatives (Figure 3A) are dominated by typical loss of 120 amu (*m/z* 245) whereas the major product ion *m/z* 215 of *glyco*-tetrahydro-b-carbolines results from the loss of a C$_5$H$_{10}$O$_5$ moiety (Figure 3B).
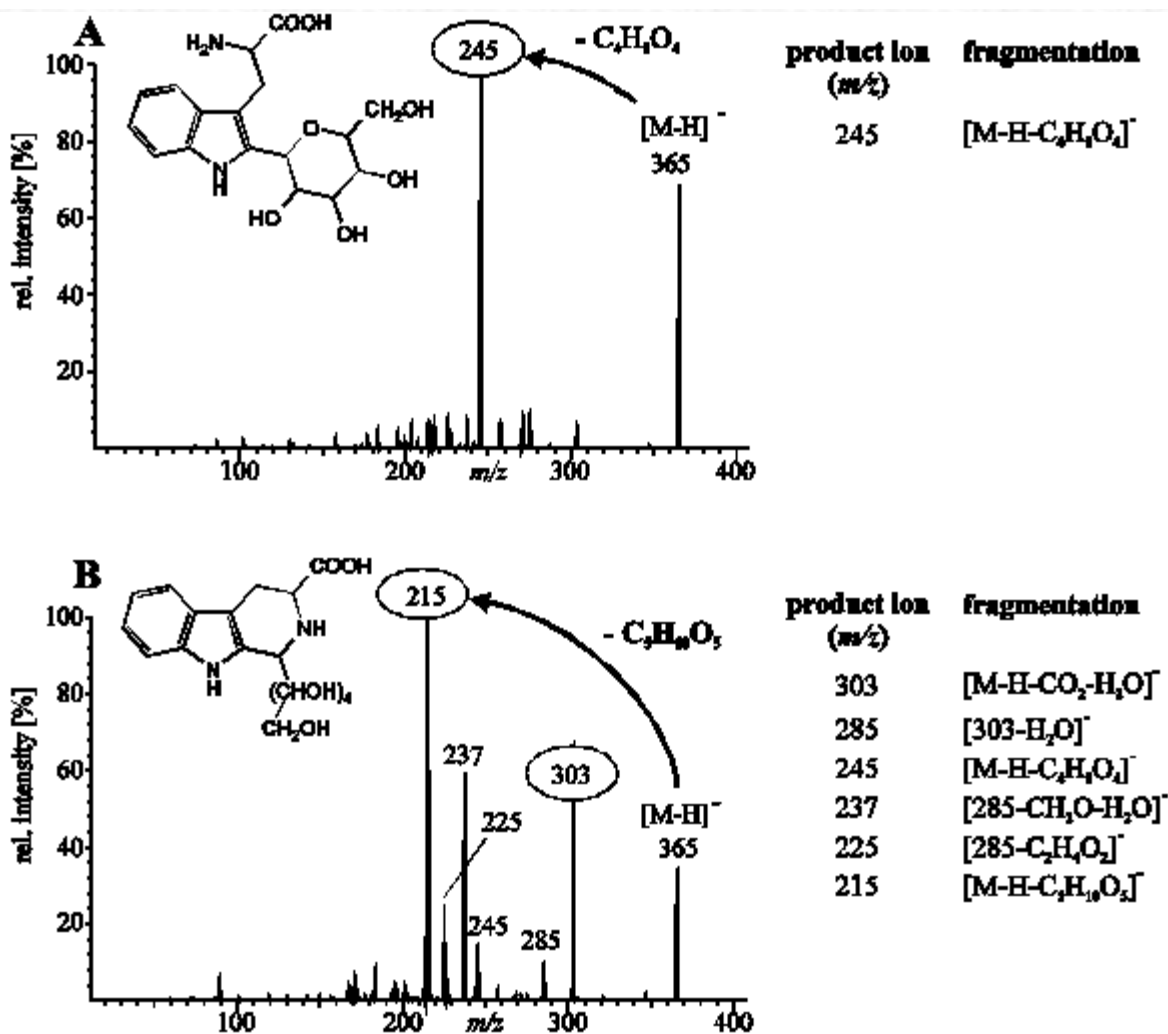
Figure 3. Product ion spectra of tryptophan glycoconjugates, precursor ions *m/z* 365 [M-H]⁻ (negative mode ESI, 19 eV; 267 mPa Ar); A: tryptophan-*C*-glucosyl derivatives; B: *gluco*-tetrahydro-b -carbolines.

These characteristic fragmentation patterns enabled us to differentiate tryptophan glycoconjugates in complex samples by HPLC-ESI-MS/MS according to their linkage type. In order to clarify the relevance of the newly identified tryptophan glycoconjugates, several food samples including seasoning sauces, alcoholic beverages and fruit products were analysed by means of HPLC-ESI-MS/MS. For these studies, limits of detection of about 100 ng/mL were achieved using selective reaction monitoring (SRM). A typical HPLC-MS/MS chromatogram of a soy sauce sample is shown in Figure 4.
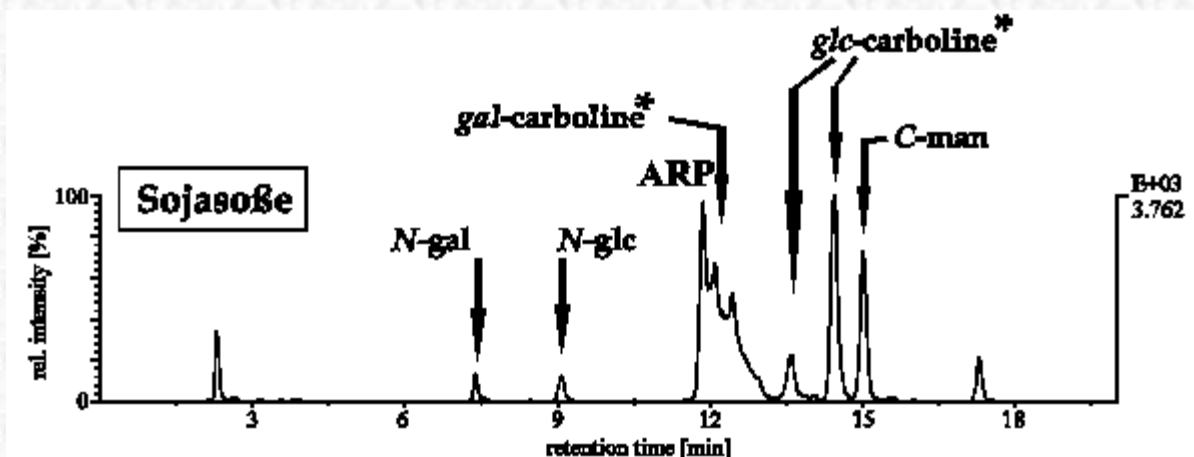
Notably, most food samples analysed contained complex mixtures of tryptophan glycoconjugates with concentrations ranging from 100 ng/mL to more than 10 m g/mL. Both, product pattern and concentration demonstrate, that the chemical condensation reaction with aldohexoses can count responsible for modification of up to 30 % of available tryptophan in nutritionals samples.

ENZYMATIC TRYPTOPHAN GLYCOSYLATION IN PLANTS

During HPLC-MS/MS analysis of food samples, the observation of one distinct tryptophan-N-glycoside in fruit syrup, which was sold as natural sweetener, attracted our attention. Consequently, we studied the occurrence of tryptophan glycoconjugates in plants and detected a single glycosylation product in numerous fruit extracts and commercially available fruit juices (Figure 5) in concentrations between 0.1 mg/L and > 10 mg/L (Table 1).
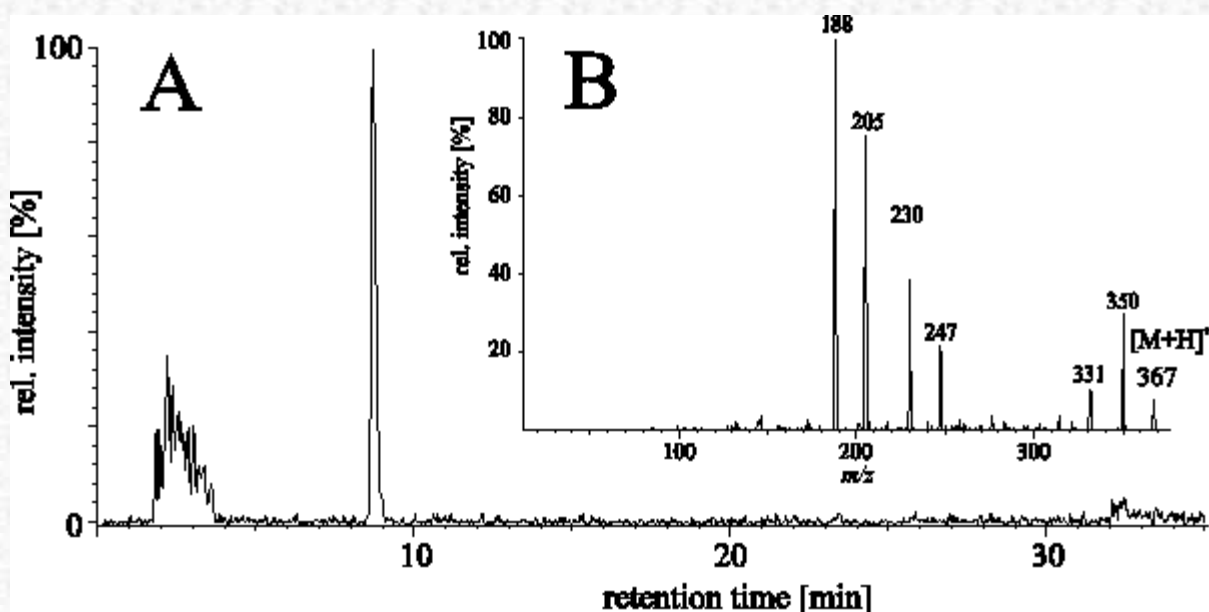

Figure 5. HPLC-MS/MS analysis of pear juice. A: mass chromatogram of m/z 367 ([M+H]$^+$ of tryptophan-hexosides). B: product ion spectrum of tryptophan-N-glucoside in pear juice (precursor ion m/z 367, 20 eV, 267 mPa Ar).

Table 1. Tryptophan-N-glucoside in fruits and fruit juices.

n[a]	sample	tryptophan-N-glucoside [mg/L]
3	pear juice	13.5 ± 1.7
2	pear fruit	7.3 ± 1.4
2	apple juice	0.7 ± 0.2
1	apricot fruit	0.3 ± 0.0
3	apple fruit	0.2 ± 0.1
2	peach fruit	0.2 ± 0.0
2	raspberry fruit	0.2 ± 0.0

1	kiwi fruit	> 0.1
1	plum fruit	> 0.1

The product ion spectrum obtained by low-energy CID of the molecular ion m/z 367 $[M+H]^+$ is dominated by the fragment ions m/z 205 and m/z 188 corresponding to loss of an intact anhydro-sugar moiety $C_6H_{10}O_5$ and subsequent loss of NH_3. As mentioned above, this fragmentation pattern is characteristic for N-glycosidically linked sugars [10], an assignment that is further supported by the retention time of the compound during HPLC analysis. Yet, information about configuration and conformation of the sugar residue is not readily available from the product ion spectra. Motivated by the adequate concentration of tryptophan-N-glycoside in pear juice, we started the isolation from this material. HR-ESI-MS analysis confirmed the molecular formula $C_{17}H_{23}N_2O_7$ for the molecular ion m/z 367 $[M+H]^+$.

The structure of the purified compound was deduced by ^{1}H, HH-COSY and ^{13}C NMR spectroscopy [8]. The results are in good accordance with those of the N-glucoside which was prepared chemically [7]. Thus, the identity of the isolated compound was unambiguously established as N^1-(b-D-glucopyranosyl-$^4C_1$)-L-tryptophan (Figure 6).
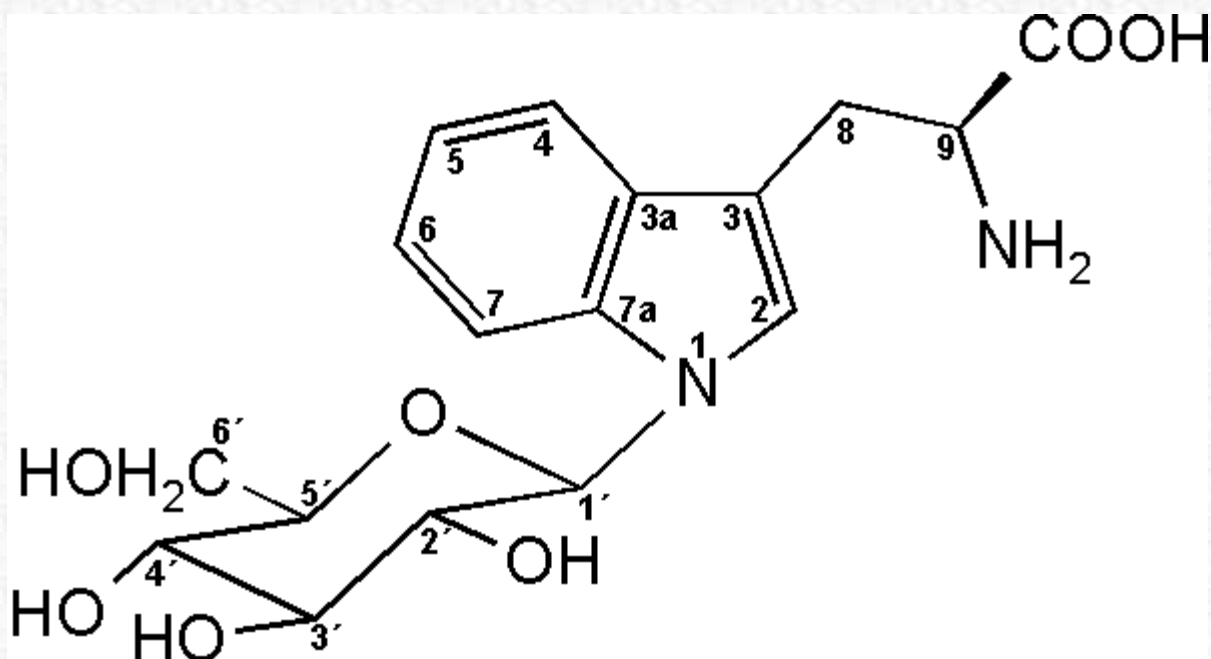

Figure 6. N^1-(b-D-glucopyranosyl-$^4C_1$)-L-tryptophan.

Previous investigation of reactions of tryptophan with aldohexoses had revealed that temperatures above 50 °C and acidic pH were a prerequisite for formation of tryptophan-N-glycosides. Furthermore, by the chemical condensation reaction C-glycosyl conjugates and $glyco$-tetrahydro-b-carbolines were always generated together with N-glycosides [7]. Therefore, occurrence of a single N-glucoside in fruits lacking any heat treatment strongly suggested the specific enzymatic biosynthesis in the plant. To obtain deeper insight into the origin of the novel glycoconjugate, we studied formation of deuterium-labeled tryptophan glycoderivatives. To select appropriate ions for these SRM experiments we performed model reactions, heating L-[indole-d_5]-tryptophan with D-glucose to 80 °C at pH 1 for 12 days. As expected, MS analysis of the model reactions yielded the d_4-C-glucosyl conjugate and d_4-$gluco$-tetrahydro-b-carbolines detected by their molecular ions m/z 371 $[M+H]^+$ as result of the loss of the deuterium at indole C-2 during the condensation reaction. However, for the chemically prepared N-glucoside molecular ions m/z 371 $[M+H]^+$ (d_4-tryptophan-N-glucoside) besides m/z 372 $[M+H]^+$ (d_5-tryptophan-N-glucoside) were found. This can be explained by deuterium-exchange at C-2: Following reversible protonation of C-3 in strongly acidic medium, H/D-exchange can

occur by migration of deuterium from C-2 to C-3 and subsequent loss of deuterium during deprotonation [11,12]. In the product ion spectrum of the d_5-N-glucoside the prominent ions m/z 210 and m/z 192 result from loss of an intact anhydro-sugar moiety $C_6H_{10}O_5$ and subsequent loss of NH_2D. Based on the results of the model experiments with d_5-tryptophan the following ions were selected for subsequent SRM experiments: m/z 367/188 for d_0-tryptophan-N-glucoside, m/z 372/192 for d_5-tryptophan-N-glucoside and m/z 371/251 for the d_4-tryptophan-C-glucosyl derivative and d_4-*gluco*-tetrahydro-b-carbolines.

To investigate formation of deuterium-labeled tryptophan glycoconjugates in pear fruits, we injected L-[indole-d_5]-tryptophan subepidermally into the fruits. After storage at room temperature for 2 to 15 days, the fruit extracts were prepared as usual and analysed by HPLC-MS/MS. These experiments exclusively proved formation of the corresponding d_5-tryptophan-N-glucoside, while the presence of any chemically formed d_4-C-glucosyl conjugate and d_4-*gluco*-tetrahydro-b-carboline could be excluded conclusively (Figure 7).
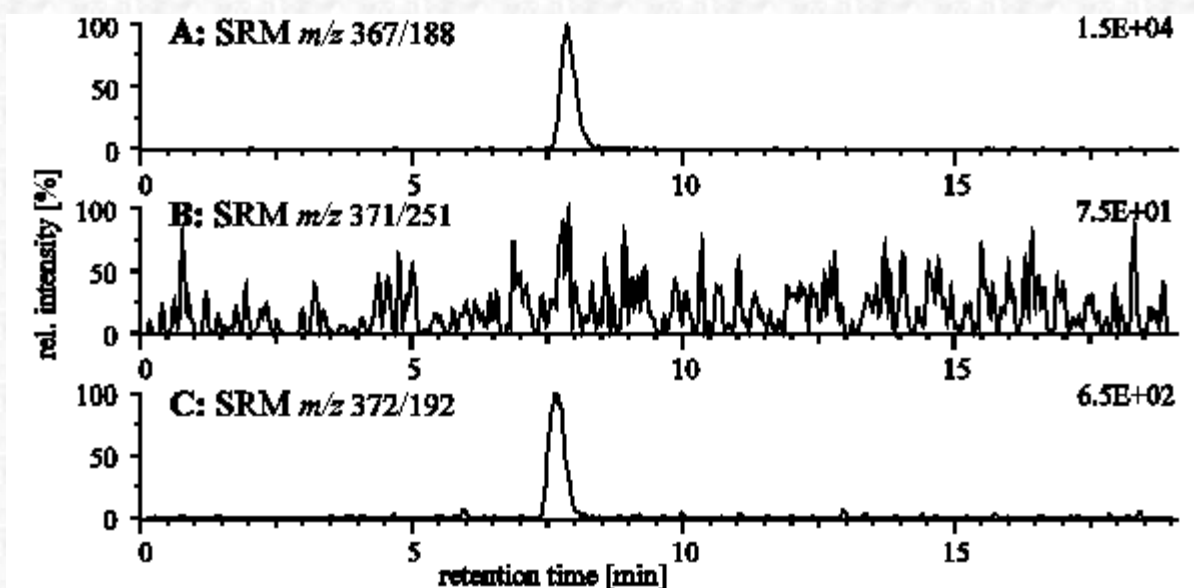

Figure 7. HPLC-MS/MS analysis of pear juice obtained after application of d_5-tryptophan. Mass chromatograms A: for d_0-tryptophan-N-glycoside. B: for d_4-C-glycosyl conjugate and d_4-*glyco*-tetrahydro-b-carbolines. C: for d_5-tryptophan-N-glycoside.

To rule-out non-enzymatic condensation of tryptophan and glucose during storage of fruits, we also incubated d_5-tryptophan in a model juice containing 2.2 % glucose, 6 % fructose and 1.1 % sucrose with a pH of 3.8 (adjusted with malic acid). The sugar content and pH value were adjusted according to data published for pear fruits [13]. Again, in " pear model juice " no formation of deuterium-labeled tryptophan glycoconjugates was observed. Finally, we compared the mass spectra of deuterated N-glucoside obtained from chemical condensation with that of the deuterated N-glucoside found in pear juice after application of d_5-tryptophan (Figure 8).
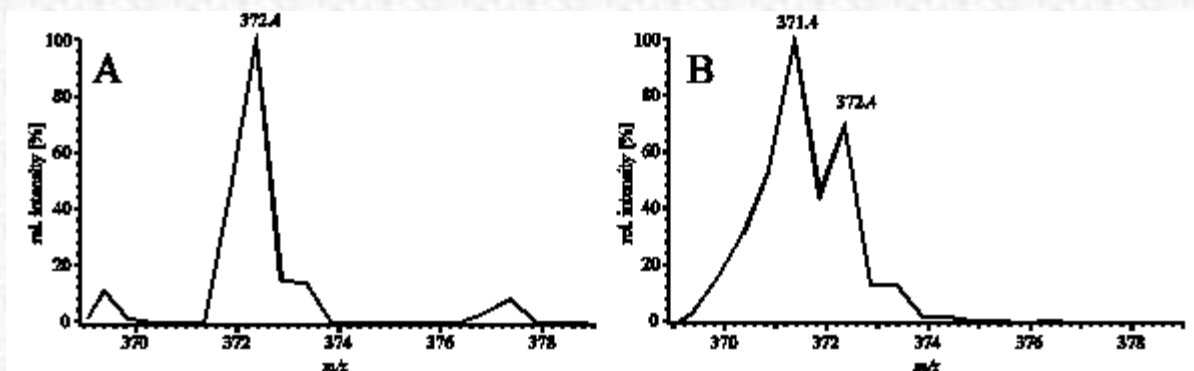
Figure 8. ESI-MS spectra showing molecular ions of tryptophan-*N*-glucosides. A: d_5-tryptophan-*N*-glucoside formed enzymatically in pears after application of d_5-tryptophan. B: d_4-/d_5-tryptophan-*N*-glucoside formed chemically.

As mentioned above, the chemically formed *N*-glucoside yields the molecular ions *m/z* 371 [M+H]$^+$ and *m/z* 372 [M+H]$^+$ due to deuterium exchange. In contrast, the *N*-glucoside in pear juice exclusively shows the molecular ion *m/z* 372 [M+H]$^+$ as result of enzymatic biosynthesis which yields d_5-tryptophan-*N*-glucoside from the labeled precursor in a highly specific manner. Taken together, our data convincingly demonstrate the enzymatic formation of the *N*-glucoside from the amino acid tryptophan in pear fruits.

As conclusion, this is the first report on naturally occurring N^1-(b-D-glucopyranosyl-$^4C_1$)-L-tryptophan representing a novel intermediate of tryptophan metabolism in plants. Future research will establish the potential of this compound to serve as a marker substance for authenticity control and will provide a reliable analytical basis for the detection of adulteration and processing of fruit products. Furthermore, the identification of this particular tryptophan metabolite provides the means to study enzymatic *N*-glycosylation reactions in plants [14] in greater detail.

ENZYMATIC TRYPTOPHAN GLYCOSYLATION IN MAN

Following the detection of tryptophan glycoconjugates in food as reported above, we analysed human urine for the occurrence of these tryptophan derivatives. By applying HPLC-ESI-MS, unexpectedly a single tryptophan glycoconjugate was detected (Figure 9).
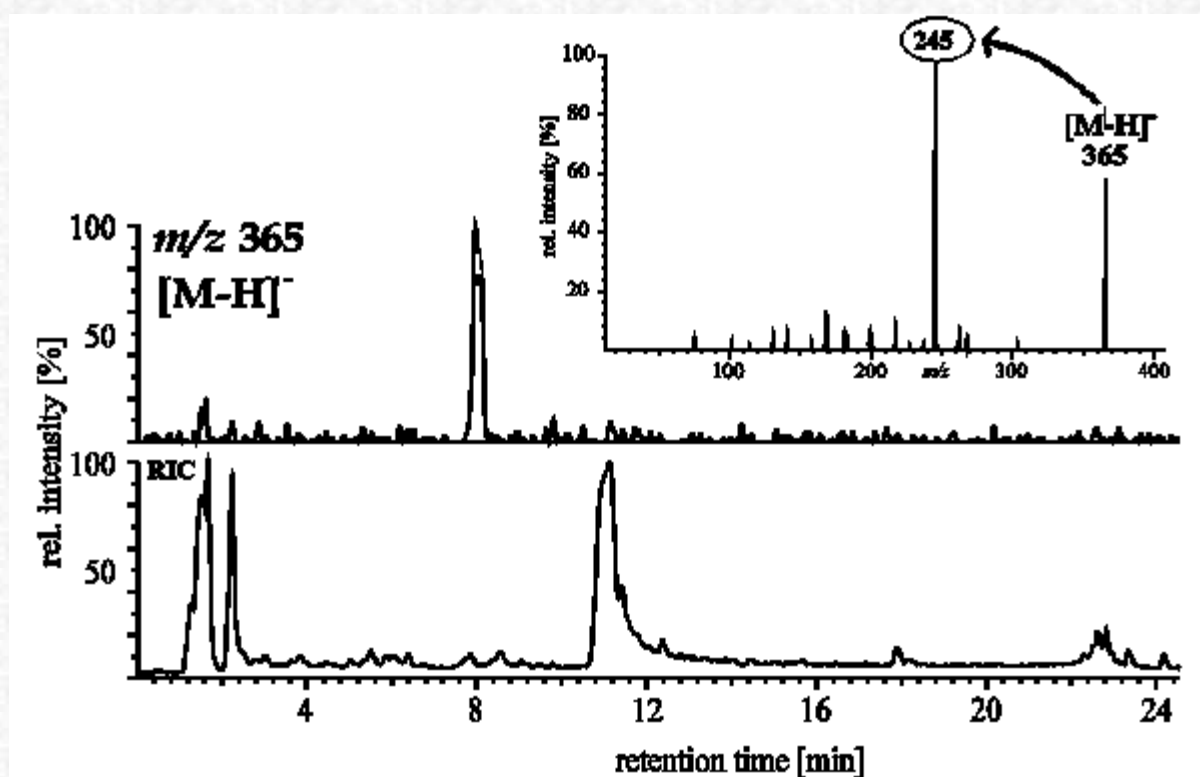

Figure 9. HPLC-MS/MS analysis of human urine. A: mass chromatogram of *m/z* 365 ([M-H]$^-$ of tryptophan-hexosides). B: product ion spectrum of *C*-glycosyl derivative in human urine (precursor ion *m/z* 365, 20 eV, 267 mPa Ar).

The product ion spectrum as obtained by low-energy CID of the protonated molecule (ESI positive mode) shows a fragmentation pattern dominated by the loss of 120 amu, corresponding to the cleavage of a $C_4H_8O_4$-moiety characteristic for *C*-glycosyl derivatives and *glyco*-tetrahydro-b-carbolines. Low energy CID of the deprotonated molecular ion (ESI negative mode) yields a product ion *m/z* 245 again resulting from the characteristic loss of a C H O -moiety (Figure 9). Thus, the compound in human urine convincingly proved to be a *C*-glycosyl derivative of

tryptophan. To further characterize the sugar residue of the glycoconjugate, we verified the structure by ^{1}H- and ^{13}C-NMR spectroscopy as well as by HH-COSY, HMQC and HMBC experiments. Furthermore, NOE experiments and acid hydrolysis followed by GC-MS analysis of MOA derivatives enabled us to determine configuration and conformation of the hexopyranosyl moiety. Finally, the human metabolite was identified as 2-(a -mannopyranosyl)-L-tryptophan preferentially adopting a $^1C_4$-conformation (Figure 10) [7].
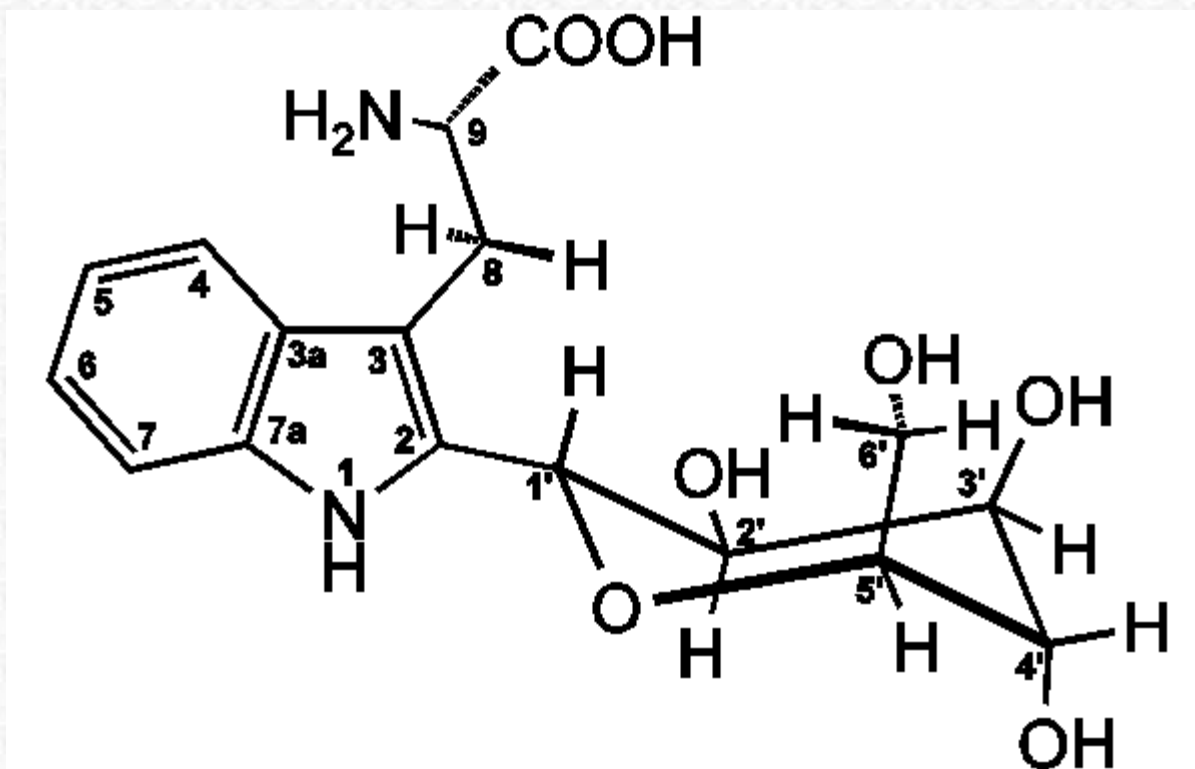

Figure 10. 2-(a -Mannopyranosyl)-L-tryptophan in human urine.

Thus, the structure of the carbohydrate moiety is consistent with the protein-bound 2-(a -mannopyranosyl)-moiety in RNase [1-3]. Concerning formation of the novel tryptophan metabolite, one has to consider enzymatic glycosylation of free tryptophan as well as degradation of proteins containing mannosylated tryptophan residues. Interestingly, Hofsteenge and coworkers reported on a novel posttranslational modification of tryptophan residues in proteins identifying the *C*-glycosidic attachment of a -D-mannopyranose to the C-2 of the indole ring of Trp^7 in human RNase [1-3]. This novel kind of protein glycosylation proved to be enzyme catalyzed employing dolichyl phosphate mannose as a precursor [4,5]. Recent studies revealed that the *C*-mannosylation of tryptophan residues is a rather common modification and occurs for example in four proteins of the human complement system: in the complete " membrane attack complex " 50 of the 113 tryptophan residues were found to undergo *C*-mannosylation [6]. Still, clarification of the mechanism of the biosynthesis of 2-(a -mannopyranosyl)-L-tryptophan as well as its role in human metabolism requires further study.

CHEMICAL *VERSUS* ENZYMATIC GLYCOSYLATION OF TRYPTOPHAN

Applying extensive HPLC-MS/MS analysis, our studies on glycosylation of tryptophan clearly demonstrate the reactivity of the indole moiety towards modification by carbohydrates. In plant-derived nutritional sources up to 30 % of the available amino acid can be converted into the respective glycoderivatives by non-selective chemical condensation reactions. Interestingly, in numerous fruits selective and enzyme-catalyzed glycosyl transfer was observed, yielding exclusively N^1-(b-D-glucopyranosyl-$^4C_1$)-L-tryptophan. Likewise, in humans specific formation of the novel *C*-glycosyl derivative 2-(a -mannopyranosyl-$^1C_4$)-L-tryptophan underlines the relevance of specific glycosylation reactions in biological systems. As conclusion, structural identification of both metabolites in plants and

humans stimulates further studies on biosynthesis and function of tryptophan glycoconjugates.

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