Scientific Research Publishing

American Journal of Plant Sciences, 2019, 10, 1196-1205
http://www.scirp.org/journal/ajps
ISSN Online: 2158-2750
ISSN Print: 2158-2742

Isolation and Characterization of Glycopeptides with Analgesic Activities from *Marasmius androsaceus*

Xianling Yang[1], Duoduo Xu[2], Qipin Gao[2], Yang Gao[2*]

[1]Jilin Institute for Drug Control, Changchun, China
[2]Changchun University of Chinese Traditional Medicine, Changchun, China
Email: *gaoqipin@sina.com

How to cite this paper: Yang, X.L., Xu, D.D., Gao, Q.P. and Gao, Y. (2019) Isolation and Characterization of Glycopeptides with Analgesic Activities from *Marasmius androsaceus*. *American Journal of Plant Sciences*, **10**, 1196-1205.
https://doi.org/10.4236/ajps.2019.107085

Received: May 30, 2019
Accepted: July 26, 2019
Published: July 29, 2019

Abstract

To screen and characterize the analgesic components from *Marasmius androsaceus*, the water-soluble extracts from the mycelium of *M. androsaceus* were isolated by ethanol precipitation followed by macroporous resin chromatography and activity-based fractionation. Analgesic activities were found to be associated with glycopeptides. The glycopeptide fractions were further purified by gel-permeation chromatography and two purified glycopeptides, D2H, D2L, were obtained. Next, the physicochemical properties and characteristics of these purified glycopeptides were studied. They were composed of glucose and mannose and 10 different amino acids and with molecular weights of 18,000 and 8000 Da, respectively. The glycosidic linkages in the purified glycopeptides were analysed via methylation analysis. The sugar portion of the glycopeptides was composed of 1 - 4 linked Glc as the main chain with side chains composed primarily of Man linked to the 6 position of the main chain; the non-reducing terminal was composed of Glc. The glycosidic band in the main chain was identified as being in the β-configuration by the IR absorbance at 891 cm^{-1}.

Keywords

Characterize, Analgesic Components, *Marasmius androsaceus*

1. Introduction

M. androsaceus is a fungus that belongs to the order Agaricales also known as the small white mushroom *Branch Marasmius*. As a Chinese medicine, *M. androsaceus* has analgesic properties [1] and antihypertensive effect [2]. The ex-

tract from the mycelium of *M. androsaceus* had been developed into a new drug "An Luotong" in China to treat pain caused by rheumatism, which seriously affects both the function and life quality of the patients. The chemical composition of the mycelium of *M. androsaceus* has been found to primarily contain mannitol, cholesteryl, amino acids, hydroxyl-cinnamic acid, ergosterol, carbohydrates, proteins and organic acids and sterols. Organic acids and sterols had been considered to be the analgesic component [3] [4]. However, the organic acids and sterols in the mycelium of the fungus are very low in quantity and are water-insoluble. In clinical use, however, "An Luotong" is prepared from water-soluble fraction of the mycelium of *M. androsaceus* [5]. All together, the active components in "An Luotong" are unlikely to be organic acids and sterols, thus remain to be elucidated. Toward this end, as we reported herein, we separated and screened the water extract from the mycelium of *M. androsaceus* for the effective components, and subsequently, purified the active components to study their physicochemical properties and chemical characteristics. This work thus allows the further development of *M. androsaceus.*

2. Results

2.1. Screening and Analysis of the Water Extract

The water extract from the mycelium of *M. androsaceus* was divided into three parts: the water-insoluble part (R), ethanol-insoluble part (P), and ethanol-soluble part (S); the recovery yield for each part was 23%, 28% and 45%, respectively. The animal experiments showed that, compared to the positive control with identical dosages, sample P had superior analgesic activity, whereas samples R and S showed no improved activity. P contained 66.1% carbohydrate, 35.3% protein and 2.1% uronic acid and ranged in molecular weight from 850 to 20,000 Da. These compounds were not detected in sample R because of poor water solubility. S contained approximately 10% carbohydrate and 15% protein and was mostly soluble in ethanol. Sample P was treated via the Savage method several times, which did not reduce the protein content relative to the initial sample. Furthermore, gel column chromatography on Sephadex G-150 indicated that the elution curves of the carbohydrate and protein overlapped significantly in sample P (**Figure 1**). These results indicated that P was composed primarily of glycopeptides, which impart the analgesic effects of *M. androsaceus.* Next, the component sugars of sample P were investigated by gas chromatography, and the results are shown in **Figure 2**. The sugar portion of the glycopeptides was composed primarily of glucose (Glc) and mannose (Man) with the molar ratio of 9:1.

2.2. Fractionation of Sample P and Physicochemical Properties of D1, D2, and D3

The fractions eluted with water (D1), 30% ethanol (D2), 60% ethanol (D3), and 95% ethanol (D4), with 32%, 37%, 22%, and 2% recovery, respectively, were ob-

tained after macroporous resin chromatography from Sample P. Because the recovered amount of D4 was quite low, it was not studied further. The physical and chemical properties of the other three samples are shown in Table 1.

These samples contained neutral sugars and peptides in different proportions, but their carbohydrate portions were composed of the same type of monosaccharide.

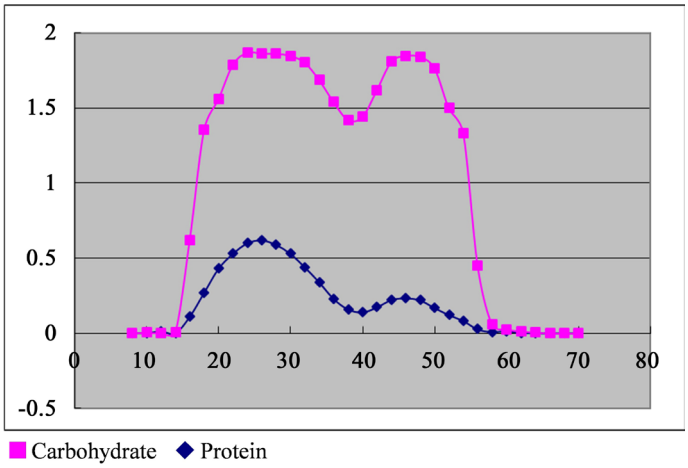

Figure 1. Gel permeation chromatography on Sephadex G-150 of Sample P.

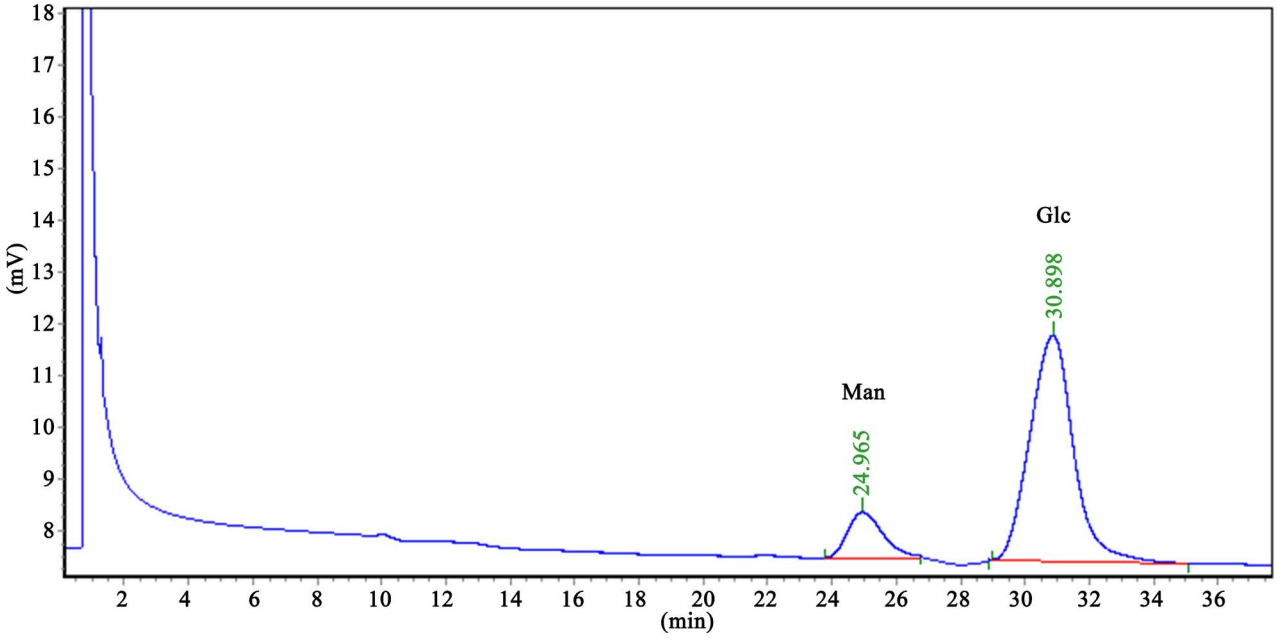

Figure 2. Component sugars analysis of sample P.

Table 1. Physicochemical properties of the different glycoproteins.

Properties Sample	Carbohydrate content (%)	Protein content (%)	Uronic acid content (%)	Component sugars (Man:Glc)	Molecular weight of main peak (D)
D1	70	37	1.9	1:10	1800
D2	63	44	1.1	1:11	15,000
D3	50	63	1.4	1:10	5700

Ten amino acids, Asp, Ser, Arg, Gly, Thr, Pro, Ala, Val, Met and Lys, were contained within the samples in different proportions, suggesting that they form a series of glycopeptides with similar structural characteristics. The animal test demonstrated that D1, D2, and D3 all have analgesic properties, and that D2 has the highest activity of the samples.

2.3. Analgesic Experiment and the Test for Maximum Administrable Amount

The analgesic experiments were performed by testing the inhibitory activity of the compounds on the writhing times of mice induced by acetic acid, and the results are listed in Table 2. The experiment demonstrated that samples P, D2, and D3 were significantly different from control group, P and D2 groups showed the highest activity compared with others samples.

Water extract and sample P were detected about their maximum administrable amount. The largest amount that could be administrated was given to mice, and no abnormality was observed withins two weeks.

2.4. Purification and Analysis of the Glycosidic Linkages in the Purified Glycopeptides

Based on the results for the analgesic effect and recovery amount, D2 was selected for further purification by gel-permeation chromatography to isolate glycopeptides with higher purity. D2 was loaded onto a Sephadex G-100 column (60 × 4 cm) and fractions were combined and lyophilised according to their elution time; two glycopeptides, D2H (fractions 12 - 25) and D2L (fractions 26 - 58), were thus obtained (Figure 3). The purity of D2 and D3 was confirmed by HPLC, each showing a single symmetrical peak; their molecular weights were determined to be 18,000 D (D2H) and 8000 D (D2L), respectively, as calculated by GPC software using the standard curve for the molecular markers of polysaccharide (data not shown).

Table 2. Inhibition Activity of writhing times induced by acetic acid in mice.

Samples	Dosages (g/kg)	Number of mice	Writhing times within 20 min
Control group	——	10	25.5 ± 4.19
Positive group 1	0.6	10	15.4 ± 1.86
Positive group 2	0.2	10	11.7 ± 2.17*
R	0.6	10	23.87 ± 3.82
P	0.6	10	12.7 ± 2.58*
S	0.6	10	20.8 ± 1.92
D1	0.6	10	16.1 ± 3.68
D2	0.6	10	12.1 ± 1.49*
D3	0.6	10	14.2 ± 1.42*

The material of "An Luotong" was used as positive control 1 and Aspirin was uesd as positive control 2; Results were presented as means ± SD (n = 10). * means $P < 0.05$ compare with control group.

2.5. Fraction in Tubes 12 - 25 and Fraction in Tubes 26 - 45 Were Pooled to Make D2H and D2L Respectively

D2H and D2L are composed of Man and Glc, and D2L also contains a small amount of Gal. The results of methylation analysis are listed in Table 3, indicating D2H and D2L have a glucan main chain consisting of 4-linked Glu. The side chain of the glucan consists of different glycosidic linked Glc and Man residues attached to the main chain at the 6 position of the 4-linked Glu; their non-reducing termini also are composed of Glc. Additionally, 2-linkage Gal is present in D2L as a side chain. The IR spectra of D2H and D2L contain absorbance peaks at 891 cm^{-1}, indicating the main chain of the glucan is linked via β-1→4 Glc (Figure 4).

3. Discussion

Glycopeptides and glycoproteins are widely present in plants and fungi and have

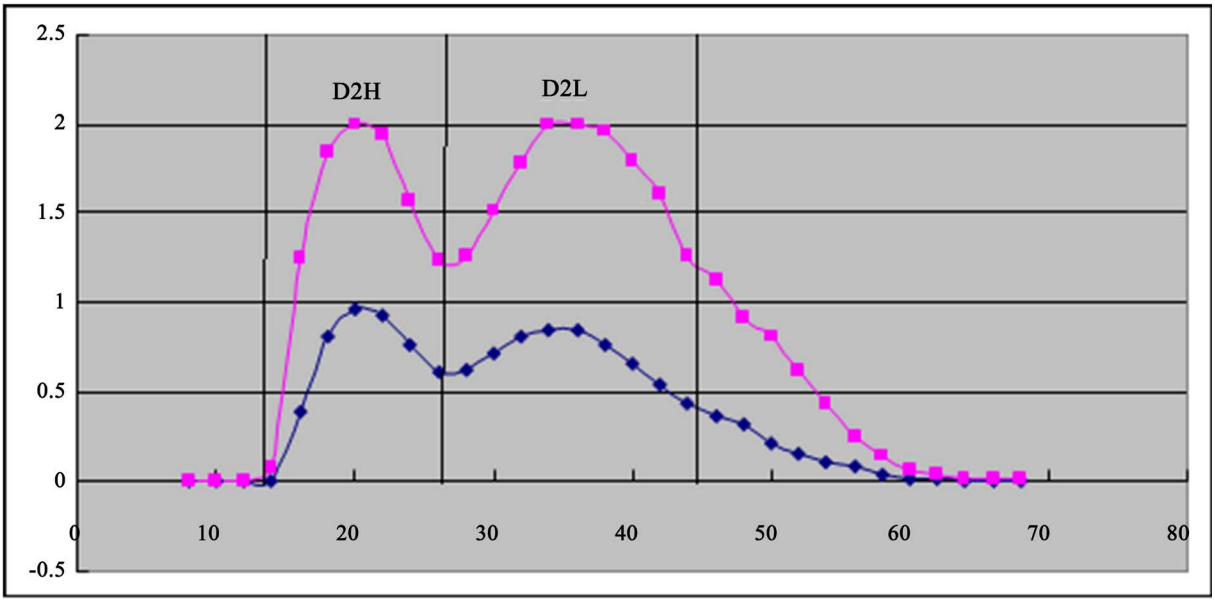

Figure 3. Purification of D2 by gel permeation chromatography.

Table 3. Analysis of glycosidic linkages of D2H and D2L.

Glycose residue	Fragment	Configure	Molar ratio (%)	
			D2H	D2L
Glc	$2,3,4,6\text{-}Me_4$	1-	8.2	10.5
	$3,4,6\text{-}Me_3$	1 - 2	7.4	6.1
	$2,4,6\text{-}Me_3$	1 - 3	4.2	7.9
	$2,3,6\text{-}Me_3$	1 - 4	47.3	42.8
	$2,3,4\text{-}Me_3$	1 - 6	21.5	15.6
	$2,3\text{-}Me_2$	1 - 4, 6	5.3	6.4
Man	$2,3,6\text{-}Me_3$	1 - 4	2.6	3.5
	$2,6\text{-}Me_2$	1 - 3, 4	3.8	4.7
Gal	$2,3,6\text{-}Me_3$	1 - 2	—	2.6

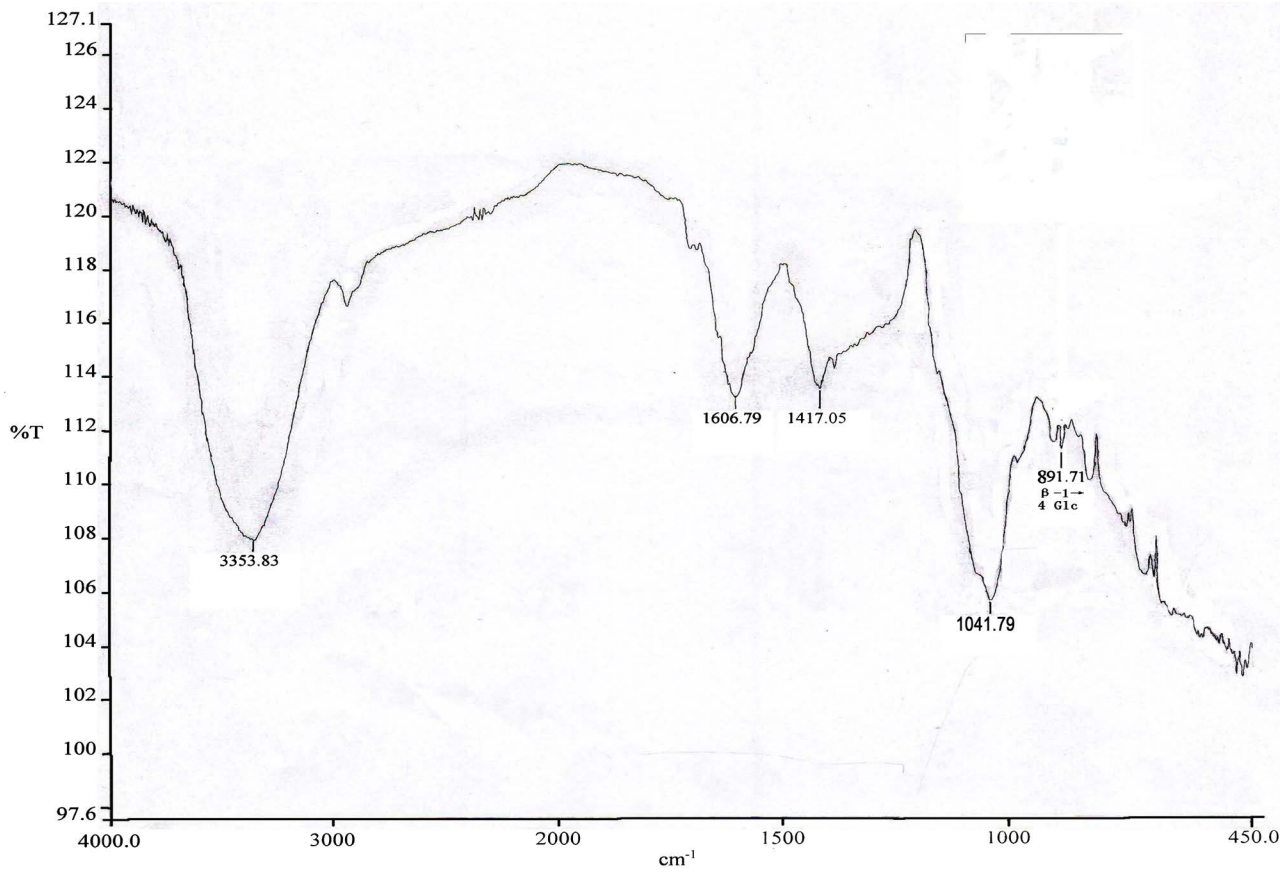

Figure 4. IR spectrum of D2.

been found to possess myriad pharmacological properties, such as immunomodulatory, anti-tumour and hypoglycaemic activities [6] [7] [8] [9]. It was also reported that glycopeptides from Ganoderma show analgesic activity [10] [11] [12].

An luotong, which is a traditional medicine and prepared from the mycelium of *M. androsaceus*, is used in China as an analgesic. But its chemical composition and active components have not been elucidated; hence there is no method for quality control and poor understanding of its modes of action. Herein, our work has shown that the water extract of the mycelium from *M. androsaceus* contains approximately 30% glycopeptide, which was the primary analgesic material. The glycopeptides in *M. androsaceus* were composed of a series of similar glycopeptides that all had analgesic properties. The D2 fraction, isolated by macroporous resin chromatography, showed the highest activity of the two glycopeptides purified by gel-permeation chromatography. D2H and D2L contained Glc, Man and a small amount of Gal as well as ten different peptides in different proportions. Methylation analysis indicated that D2H and D2L have a glucan main chain consisting of β-1→4 Glc and a side chain consisting of Glc and Man residues attached at the 6 position of the β-1→4 Glc in the D2H and D2L. Additionally, the glycopeptides were partially terminated with glucose residues, and the peptide chains were linked to the complex carbohydrate moiety at the terminus. These results indicated that the analgesic effect may come from

the common structural elements of these glycopeptides and their biological effects may associated with the inhabiting release of inflammatory, such as cytokines, and prostaglandins [13].

In summary, our results and finding are useful for better quality control as well as understanding the structure-activity relationships and underlining mechanisms in the future.

3.1. Methods

3.1.1. Reagents and Materials

The mycelium extract of *M. androsaceus* was supplied by Jilin Xinhua Medicine Ltd. (Jilin City, Jilin Province, China) Sephadex G-100, G-150, DEAE-Sephadex G-50; standard monosaccharide and BSA were obtained from Sigma. Protein Assay Reagents A and B were prepared in our laboratory. D101 macroporous resin was purchased from Tianjin University. All other chemicals were from domestic sources. "An Luotong" and Aspirin were purchased from the market of China.

3.1.2. Preparation of Samples

The mycelium of *M. androsaceus* was extracted with water for 3 h at 100˚C. After centrifugation, the solid residuals were collected by centrifugation and freeze-dried to afford the water insoluble fraction (R). The aqueous solution was concentrated to small volume and then deposited with 80% ethanol; after centrifugation, the precipitated material was lyophilised to afford the ethanol-insoluble portion (P), and the ethanol supernatant was evaporated to yield the ethanol-soluble portion (S).

3.1.3. Analysis of Physicochemical Properties

The total carbohydrate, uronic acid and protein concentrations were quantified using phenol-sulphuric acid [14], m-hydroxyldiphenyl [15] and Bio-Rad protein methods, respectively, with glucose (Glc), glucuronic acid (GlcA), and bovine serum albumin (BSA) as standards, respectively. The molecular weight of each sample was analysed by High Performance Liquid Chromatography (Shimadzu LC-2010) using an OH-park column equilibrated with 0.7% sodium sulphate, and the calibration curves were obtained using a dextran standard; the molecular weight of each sample was calculated by GPC software from the National Institute for the Control of Pharmaceutical and Biological Products of China. The sugar components were analysed by converting them to alditol acetates, which were detected by GC [16]. GC was performed at 180˚C using a Fuling GC-9790 gas chromatography instrument equipped with a flame-ionisation detector and glass column (i.d.: 3 mm; L: 200 cm) packed with 1% OV-225 controlled by a uniport HP and N-2000 data station. The amino acids were analysed using an S-433D (Germany Sykam) automatic amino acid analyser.

3.1.4. Inhibition Activity by Acetic Acid Leading to the Writhing Response in Mice and Actual Toxicity Test

The writhing test was used to evaluate analgesic activity according to Koster *et*

al. [17]. The samples used in the experiment were prepared in our laboratory. The mice (weight 18 - 22 g, certificate of conformity SCXK: 2008-0005 obtained from the College of Pharmacy at Jilin University) were divided into equal groups. Samples and An Luotong (positive 1) and Aspirin (positive 2) dissolved in water were administered orally once a day for 10 days during normal meal times; the control group was given the same volume of pure water (0.2 mL). Aspirin was used as another positive control and was given the mouse only in the last administration. Each mouse was injected intraperitoneally with 0.6% acetic acid (0.2 mL) 1 hour after the last sample dosage, and writhing times for the mice of less than 20 minutes were observed. All data were expressed as mean ± SE. The statistical significance of any difference in each parameter among the groups was evaluated by using one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD) comparison tests for Post hoc t-tests, Differences of $P < 0.05$ were considered statistically significant.

Before the analgesic experiment, the 20 mice were administrated orally the mycelium extract of *M. androsaceus* and the sample P (100 mg/mouse), to detect its maximum administrable amount since LD50 could not be detected and the mice were observed for two weeks continuously.

3.1.5. Macroporous Resin Chromatography

The P samples (30 g) were dissolved in water (100 mL) and loaded onto a macroporous resin column (100 × 10 cm) and eluted with water (D1), 30% ethanol (D2), 60% ethanol (D3), and 95% ethanol (D4). Different fractions were collected, concentrated and lyophilised to obtain D1, 2, 3, and 4.

3.1.6. Purification of D2

D2 (200 mg) was further subjected to gel-permeation chromatography on a Sephadex G-100 (60 cm × 4 cm) column eluted with water, and fractions were collected every 6 mL. The sugar and protein concentrations were determined using the phenol-sulphuric acid and UV absorbance at 280 nm methods, and fractions were combined according to their elution curves to yield D2H and D2L fractions and freeze-dried.

3.1.7. Glycosidic Linkage Analysis of the Purified Glycopeptides

Glycosidic linkage analysis was carried out via the Hakomori method [18] by GC (Shimadzu GC-2010) and GC-MS (Agilent 6890N-5973) using a DB-1 capillary column (30 m × 0.25 mm). The injector temperature was 250˚C and the column temperature was held at 60˚C for 5 min and then increased to 220˚C at 5˚C/min. The samples were dissolved in acetone, and the injected volume was 1 μL. The molar ratios were calibrated based on the peak areas and response factors [19] of the flame ionisation detector. The mass spectra were recorded in the positive ion electron ionisation (EI) mode using an MSD ChemStation. The FT-IR spectrum were acquired using Bruker (Germany) Vertex 70 FTIR. The samples were pressed into KBr pellets and the spectra were recorded in the transmittance mode over the frequency range of 4000 and 400 cm^{-1}.

Acknowledgements

We thank Jilin Xinhua Medicine Ltd. for offering the mycelium extract from *M. androsaceus* and Prof. Zhiqiang Liu, Changchun Institute of Applied Chemistry at the Chinese Academy of Sciences, for the amino acid analysis. The project was supported by National Science Foundation of China (No. 30873370).

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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