Available online at www.sciencedirect.com
ScienceDirect
Carbohydrate Polymers 69 (2007) 644–650
Carbohydrate Polymers
www.elsevier.com/locate/carbpol

Synthesis and antitumor activity of arginine–glucosamine conjugate

Li Zhang [a,b], Wanshun Liu [b], Baoqin Han [b], Dongfeng Wang [a,*]

[a] College of Food Science and Engineering, Ocean University of China, Qingdao 266003, China
[b] College of Marine Life Sciences, Ocean University of China, Qingdao 266003, China

Received 26 July 2006; received in revised form 29 October 2006; accepted 31 January 2007
Available online 15 February 2007

Abstract

Using D-glucosamine hydrochloride ($GlcNH_2$·HCl) as starting material, a new amino acid sugar conjugate, arginine–glucosamine (Arg–$GlcNH_2$), was synthesized and characterized by infrared spectroscopy, ^{13}C NMR and element analysis. Its cytotoxicity *in vitro* was evaluated by MTT assay. The inhibition ratio against human hepatoma cell SMMC-7721 was higher than that of $GlcNH_2$·HCl. This effect was accompanied by a marked increase in the proportion of S cells as analyzed by flow cytometry. In addition, SMMC-7721 cells treated with Arg–$GlcNH_2$ resulted in the induction of apoptosis as assayed qualitatively by agarose gel electrophoresis. The manner of Arg–$GlcNH_2$ cytocidal activity was concluded to be apoptosis.

Keywords: Caboxymethyl; Glucosamine; Arginine; Antitumor; SMMC-7721; Apoptosis

1. Introduction

Chitin, a poly-$\beta(1 \rightarrow 4)$-*N*-acetyl-D-glucosamine, is a natural biopolymer present in the exoskeleton of crustaceans and in cell walls of fungi, insects and yeast. A series of oligomers and monosaccharides, such as D-glucosamine hydrochloride ($GlcNH_2$·HCl) and *N*-acetyl-D-glucosamine (GlcNAc) can be obtained by either chemical or enzymatic hydrolysis of chitin and chitosan (Akiyama, Kawazu, & Kobayashi, 1995). The existence of reactive hydroxyl and amino groups offers wide possibility for obtaining new polymers. The derivatives that can be obtained are known to have a range of biological activities (Chen, Yang, & Du, 2005; Martin, Castro, Moy, & Rubin, 2003; Wang & Chen, 2005).

According to our previous study, $GlcNH_2$·HCl and $GlcNH_2$ resulted in a time and dose-dependent reduction in hepatoma cell growth, while GlcNAc did not (Zhang, Liu, Han, Peng, & Wang, 2006). Wang et al. (2003a, 2003b) reported that GlcNAc, $GlcNH_2$·HCl and $GlcNH_2$ could induce proliferation of leukemia K562 cells and make them differentiate between macrophage. $GlcNH_2$ at concentrations in a certain range could kill tumor cells without influencing normal cells (Friedman & Skehan, 1980). As it is known, L-arginine (Arg) could enhance NK and LAK cell activity, which was mediated by $CD56^+$ cells both *in vitro* and *in vivo* (Park, Hayes, Garlick, Sewell, & Eremin, 1991). Supplemental dietary Arg retarded tumor growth and prolonged median survival time in mice although the mechanisms were unclear (Reynolds et al., 1990). Moreover, argininyl–fructosyl–glucose (AFG) and argininyl–fructose (AF), which are the water extracts of red ginseng, have been proved to exhibit such biological activities such as improving microcirculation and promoting spleen cell proliferation (Zheng, Zhang, & Okuda, 1998). It is therefore postulated that a combination of $GlcNH_2$ with Arg may have the potential to kill tumor cells.

Making use of monochloroacetic acid and glyoxylic acid, carboxymethyl chitin as well as *N*,*O*-carboxymethyl chitosans have been synthesized (Muzzarelli, 1988; Nishimura, Nishi, & Tokura, 1986). These water-soluble modified biopolymers were found useful as medical aids, cosmetic ingredients and metal ion chelating agents (Chen, Du, &

* Corresponding author. Tel.: +86 532 82031575; fax: +86 532 82032468.
E-mail address: wangdf@ouc.edu.cn (D. Wang).

0144-8617/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2007.01.025

Liu, 2000; Chen, Wang, Liu, & Park, 2002). Application of the similar procedure to monosaccharide such as $GlcNH_2$·HCl should allow the further functionalization of these chitin derivatives.

The purpose of this study was to synthesize arginine–glucosamine conjugate (Arg–$GlcNH_2$), and to investigate its antitumor activity. The mechanism of the Arg–$GlcNH_2$ cytocidal activity was also discussed.

2. Experimental

2.1. Materials

$GlcNH_2$·HCl was prepared and purified (purity ⩾ 99%) by our lab according to previous methods (Ingle, Vaidya, & Pai, 1973). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Chemical Co. Bio-Gel P-4 Gel was purchased from Bio-Rad Laboratories, Inc. All other chemicals were analytical reagents.

2.2. General methods

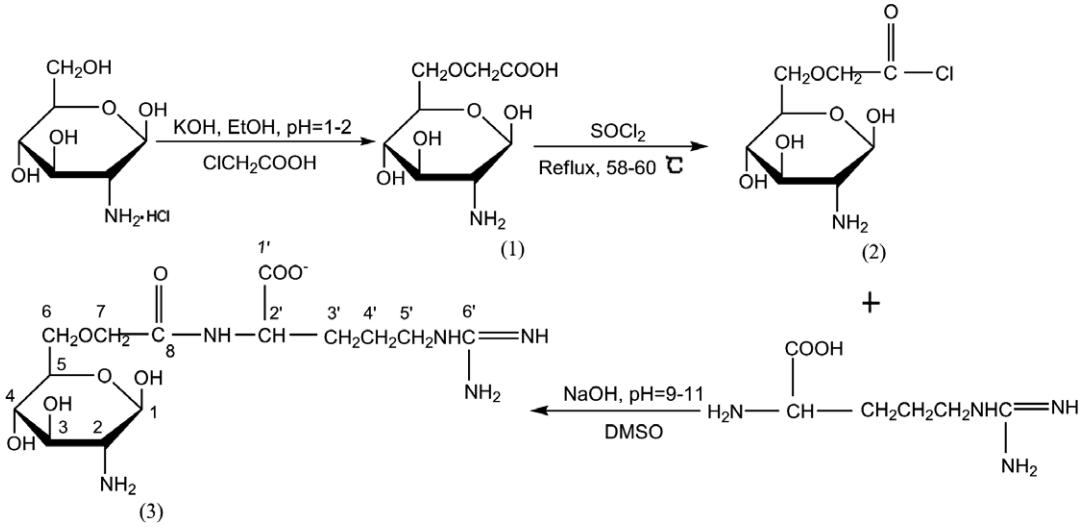
The total synthesis of target compounds was outlined in Scheme 1. The ^{13}C NMR spectrum was recorded in D_2O on a JNM ECP-600 spectrometer (JEOL, Japan) with 125 MHz at 20 °C. Melting point was measured with a BüChi Melting point B-545 (BUCHI Analytical Inc., USA). The C, H and N element content were measured by a Vario ELIII elemental analyzer (Elementar Analysensysteme GmbH). Infrared (IR) spectroscopy of the sample was recorded on a Nexue 470 FTIR (Nicolet Co., USA) in the range of 400–4000 cm^{-1}. The HPLC system consisted of a Waters 1525 pump connected to a Waters 2414 differential refractometers detector and a PC provided with Breeze 4.0 software (Waters Corporation, USA). High performance liquid chromatography (HPLC) was preformed on a Shodex SUGAR KS-801 column (300 mm × 8 mm; Showa Denko K.k., Japan). The column was kept at 40 °C with a flow rate of 0.5 ml/min.

2.3. Synthesis of CM-$GlcNH_2$ (1)

$GlcNH_2$·HCl (30 g, 0.1392 mol) was added into a three-necked flask equipped with a stirrer. It was dissolved in absolute ethanol (20 ml) under stirring to give a suspension. An aqueous solution of KOH (39 g, 0.696 mol) in absolute ethanol (150 ml) was added dropwise to the stirred solution over a period of 40 min, and the reaction mixture was stirred for another 3 h, temperature being controlled below 21 °C all along. $ClCH_2COOH$ (39 g, 0.4176 mol) was dissolved in 20 ml absolute ethanol, and the resulting solution was loaded dropwise to the flask for about 1 h, with temperature not more than 25 °C. The reaction was carried out at 21 °C for 15 h. The products were filtered and washed thoroughly with absolute ethanol to remove unreacted KOH and $ClCH_2COOH$, then dissolved in distilled water (60 ml). The pH of the solution was adjusted to 1–2. Inorganic salts (37 g) were precipitated from aqueous solution with absolute ethanol (180 ml). The filtrate was concentrated by rotary evaporator at reduced pressure below 70 °C, and then lyophilized to obtain powder as CM-$GlcNH_2$ (28 g).

2.4. Synthesis of acyl chloride of CM-$GlcNH_2$ (2)

The three-necked flask equipped with the stirrer and a reflux condenser was charged with CM-$GlcNH_2$ (8 g, 0.029 mol) and $SOCl_2$ (38 ml, 0.524 mol). The reaction mixture was allowed to stir for 8 h at 58–60 °C. After dissolution of CM-$GlcNH_2$, the $SOCl_2$ was removed by water pump.



Scheme 1. Synthesis route of Arg-Glc NH_2.

2.5. Synthesis of Arg–$GlcNH_2$ (3)

Arg (3.34 g, 0.019 mol) was dissolved in 20% solution of NaOH (10 ml) and acyl chloride of CM-$GlcNH_2$ (3.33 g, 0.013 mol) was dissolved in DMSO (5 ml). The acyl chloride and 20% NaOH were alternately added dropwise into the Arg solution under stirring to retain pH 9–11. The reaction time was 15 h, of which 2 h was spent gradually adding the corresponding components. The product was concentrated by rotary evaporation at reduced pressure below 70 °C, and precipitated by equivolume amount of acetone and absolute ethanol, then dried in vacuum at room temperature to obtain a powder (4.92 g).

The powder (1.4 g) was dissolved in distilled water, and then subjected to Bio-Gel P-4 Gel (100–200 mesh) column (1.4×120 cm) chromatography using distilled water as eluant at 15 ml/h and 3 ml per tube. Fractions eluted at 72–91.2 ml were collected, concentrated and lyophilized to give the yield of Arg–$GlcNH_2$ (0.18 g).

2.6. MTT cell viability assay

Human hepatoma cell line SMMC-7721 was procured from Shanghai Institute of Cell Biology, China. The cells were cultured in RPMI-1640 medium supplemented with 15% BCS, 100 U/ml penicillin and 100 U/ml streptomycin at 37 °C in a 5% CO_2 atmosphere. Cells were seeded in 96-well plates (Costar) at an initial density of $5–7 \times 10^5$ cells/ml, incubated for 24 h, and then treated with test compounds at final concentration of 20–1000 μg/ml respectively. Untreated cells were used as controls. The cytotoxicity of samples *in vitro* was examined with an MTT assay according to previously described procedures (Mosmann, 1983). Briefly, 40 μl MTT (5 mg/ml) solution was added to each well of 96-well plates containing $5–7 \times 10^4$ cells treated with different concentrations of samples for 96 h. The reaction was stopped after 4 h incubation by extracting the solution and adding 140 μl of 0.04 N HCl in isopropanol. The absorbance of each well was measured by an ELISA reader (Multiscan MK3, Thermo Labsystems) using a test wavelength of 490 nm. Each concentration treatment was done in triplicate wells. Then the results were expressed as the inhibition ratio. $\delta = (A - B)/A \times 100\%$, where A and B were the absorbance of the control and sample groups after 96 h incubation respectively.

2.7. DNA extraction and agarose gel electrophoresis

DNA was isolated as described previously (Sambrook & Russell, 2001). Briefly, SMMC-7721 cells treated with Arg–$GlcNH_2$ and untreated cells were collected and washed with ice-cold PBS. Then, the cells were lysed in a solution containing 50 mmol/l Tris–HCl (pH 8.0), 10 mmol/l EDTA, 10 mmol/l NaCl, 0.5% SDS and 0.5 mg/ml proteinase K and were incubated at 60 °C for 1 h. After the addition of RNase A (final concentration 0.25 mg/ml), the cells were incubated at 37 °C for 1 h. The lysate was extracted with equal volume of phenol/chloroform (1:1 v/v) and then precipitated with ethanol. The extracted DNA samples were dissolved in TE buffer and the amount of DNA equivalent to content of $3–5 \times 10^5$ cells was electrophoresed at 50 V in 1% agarose gel. The presence of DNA in the gel was visualized by ethidium bromide and was photographed under UV illumination (YLN2K, YALIEN CO.).

2.8. Cell-cycle analysis

After washing twice with PBS, cells (1×10^6) treated and untreated with Arg–$GlcNH_2$ were fixed in 70% ice-cold ethanol immediately for 4 h. Fixed cells were washed in phosphate-buffered saline with 0.1% Triton X and then stained with 20 μg/ml propidium iodide (Sigma) and 200 U/ml RNase A (Lin, Ho, & Yang, 1996). The DNA content was measured by FACS Vantage Flow Cytometer (Becton Dickinson, USA) and analyzed by ModFit LT software.

2.9. Statistical analysis

Data were presented as the arithmetic mean ± standard deviation (SD) of triplicate samples. Statistical analysis was performed using SPSS10.0. ANOVA was used to analyze statistical comparisons between groups. Differences with P-values less than 0.05 were considered to be statistically significant.

3. Results and discussion

3.1. Characterization of compounds

There were three fractions in the elution profile of the product mixture on Bio-Gel P-4 Gel column (Fig. 1). Fractions eluted at 177.6–184.8 ml and 187.2–194.4 ml were residual Arg and $GlcNH_2$ in the mixture respectively, for the elution volume of Arg and $GlcNH_2$ was 175.2–189.6 and 194.4–196.8 ml under the same elution conditions (data not shown). The HPLC shows that Arg–$GlcNH_2$ (72–91.2 ml) was completely purified from the product mixture after Bio-Gel P-4 Gel column purification. The retention time of the single peak was 9.1 min (Fig. 2).

A strong band at 1731 cm^{-1} (Fig. 3a) confirmed the high degree of carboxymethylation of CM-$GlcNH_2$, which was determined by pH potentiometric titration to be 97.45% (Muzzarelli, Tanfani, Emanuelli, & Mariotti, 1982). The substitution actually was an *O*-carboxymethylation at the C-6 position of the D-glucosamine because of the higher reactivity of the C-6 primary hydroxyl, when the degree of carboxymethylation was less than 100% (Muzzarelli, 1988). The absorption peaks in Fig. 3a at 1350–1260 and 1075–1000 cm^{-1} region, attributed to δ_{O-H} and $\upsilon_{C-O}$of –CH_2OH, respectively, nearly disappeared, compared with $GlcNH_2$·HCl. This revealed that the carboxymethylation substitution occurred at the 6-OH group (Peng, Han, Liu, & Xu, 2005). In addition, the obvious characteristic

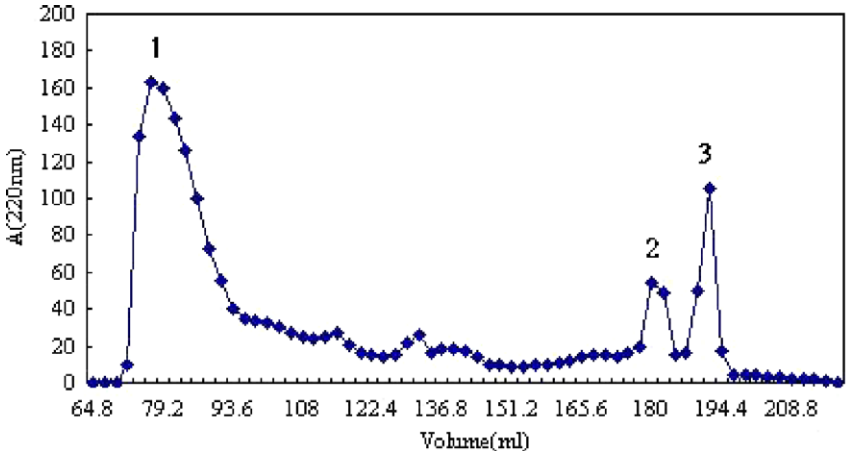


Fig. 1. The Bio-Gel P-4 column elution profile.

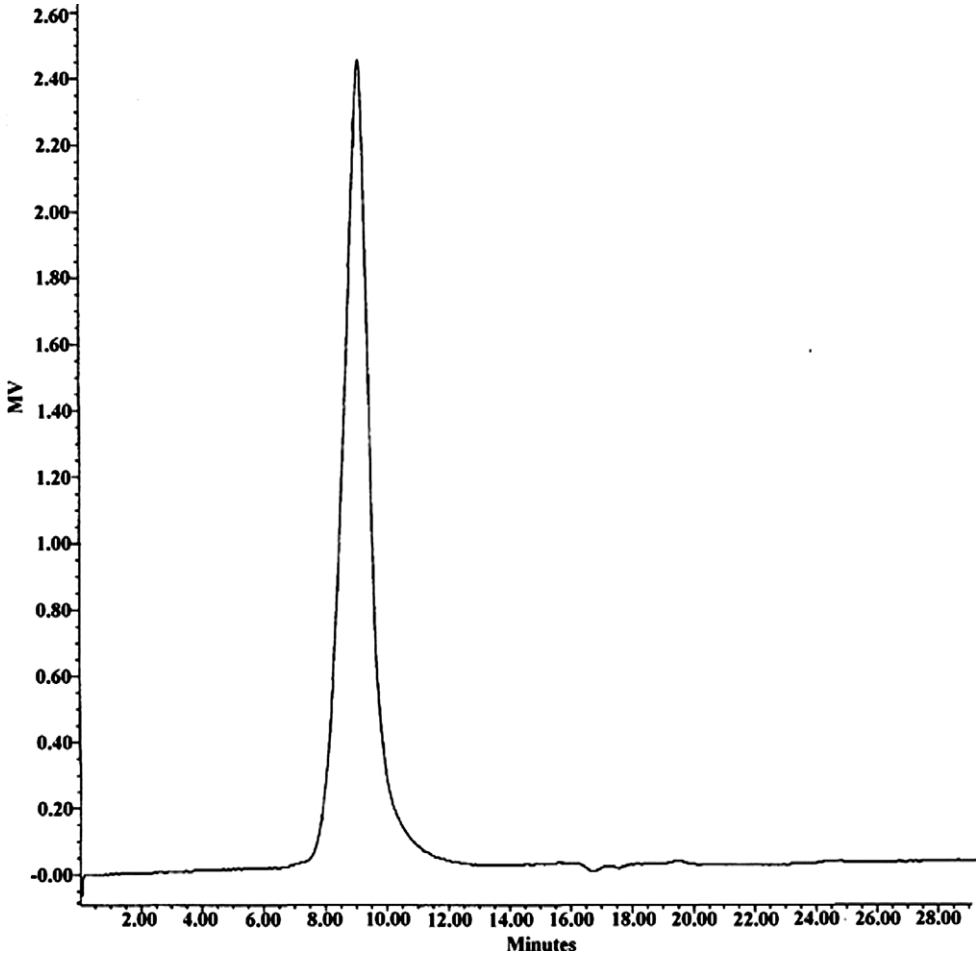


Fig. 2. HPLC trace of the Arg–$GlcNH_2$ purified from the Bio-Gel P-4 Gel column.

absorption of $-NH_2$ group at 1627 cm^{-1}(Fig. 3a) indicated that the *N*-carboxymethylation did not take place (Jin & Zhu, 1996; Yang, Du, & Qin, 2003). Fig. 3b exhibits the absorption at 1609 cm^{-1}, typical for the carbonyl stretching $\upsilon_{C=O}$ (amide I) of Arg–$GlcNH_2$ (Shigemasa, Matsuura, Sashiwa, & Saimoto, 1996). Moreover, new absorption peaks appeared at 1452 and 1377 cm^{-1} in Fig. 3b, corresponding to the C–H bending of the CH_2 group (Ning, 2000). These absorptions indicated that Arg was introduced into the D-glucosamine hydrochloride.

Fig. 4 shows the ^{13}C NMR spectra of CM-$GlcNH_2$ in D_2O. Chemical shifts were given taking DSS as reference. The signal around 173.3 ppm was assigned to the carbonyl carbon (C-8) (Zhao, Wang, Ye, & Wang, 2002). The signal

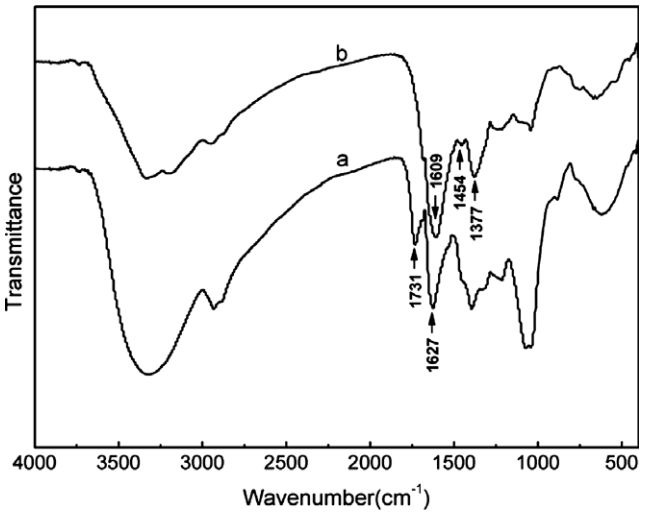


Fig. 3. FTIR spectra of (a) CM-$GlcNH_2$ and (b) Arg–$GlcNH_2$.

attributed to C-6 was split into two, one corresponding to the fraction with a free -OH group and the other to the *O*-carboxymethylated form (65.7 ppm) (Rinaudo, Dung, Gey, & Milas, 1992). Table 1 shows the ^{13}C-NMR chemical shifts of Arg–$GlcNH_2$, which agreed with the result of Zheng et al. (1996).

The melting point of Arg–$GlcNH_2$ is 250 °C (d). Anal. Calcd for $C_{14}H_{27}O_8N_5$: C, 42.73; H, 6.87; O, 32.55; N, 17.80. Found: C, 42.01; H, 6.46; O, 32.30; N, 17.63 (Wang & Wang, 2004).

3.2. Inhibitory effects of Arg–$GlcNH_2$ on the proliferation of SMMC-7721 cells in vitro

Treatment with Arg–$GlcNH_2$ for 96 h resulted in a concentration-dependent inhibition in SMMC-7721 cell growth (Fig. 5). Arg–$GlcNH_2$ exhibited much better antitumor activity than $GlcNH_2$·HCl itself did. The inhibition ratio of Arg–$GlcNH_2$ at concentration of 500 μg/ml was $60.02 \pm 4.32\%$, and reached $93.77 \pm 6.47\%$ at concentration of 1000 μg/ml, significantly higher than that of $GlcNH_2$·HCl at 500 μg/ml ($49.12 \pm 8.93\%$) and 1000 μg/ml ($80.26 \pm 4.32\%$) according to our previous report (Zhang et al., 2006). No antitumor activities were found in Arg at 100 μg/ml, though the inhibition ratio was little enhanced with increasing of concentration levels from 200 μg/ml (data not shown).

Table 1
^{13}C NMR chemical shifts of Arg–$GlcNH_2$

Positions	δ (ppm)	Positions	δ (ppm)
C-1	90.8	C-1′	173.9
C-2	60.1	C-2′	50.0
C-3	74.0	C-3′	19.5
C-4	72.3	C-4′	16.0
C-5	76.1	C-5′	40.3
C-6	63.0	C-6′	144.9
C-7	65.2		
C-8	146.8		

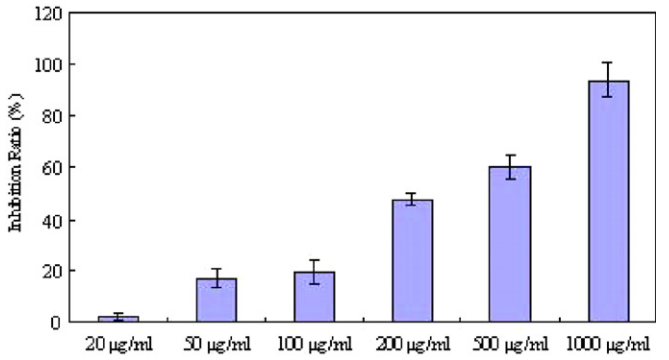


Fig. 5. Inhibition of proliferation of SMMC-7721 cells by various concentrations of Arg–$GlcNH_2$ for 96 h.

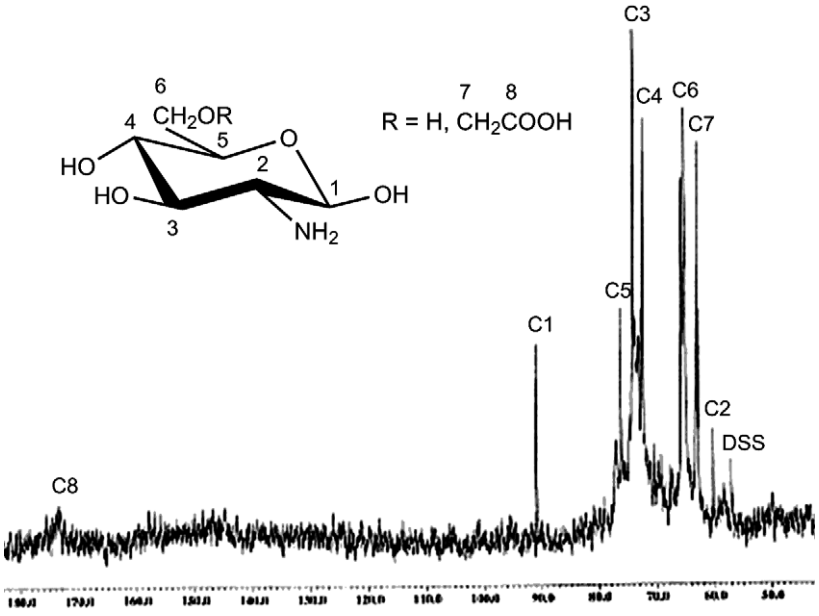


Fig. 4. ^{13}C NMR spectra (125 MHz) of CM-$GlcNH_2$ in D_2O.

3.3. Effect of Arg–$GlcNH_2$ on cell-cycle distribution of SMMC-7721 cells

As shown in Table 2, the addition of 500 μg/ml Arg–$GlcNH_2$ for 96 h significantly increased the S-phase ratio and slightly decreased the G2/M phase ratio compared with the control. Increasing of Arg–$GlcNH_2$ concentration and prolonging of treatment time increased the percentage of cells with apoptotic DNA content measurement (data not shown). The result suggested that the influence of Arg–$GlcNH_2$ on the cell-cycle distribution was a mechanism contributing to its antiproliferative effect. It is possible that Arg–$GlcNH_2$ induces S phase accumulation in SMMC-7721 cell lines by losing or inactivating the normal G1 checkpoint conferred by the retinoblastoma protein, which acts as a cell-cycle inhibitor (Murata et al., 2006; Qin, Runkel, Deck, DeDios, & Barsoum, 1997).

Table 2
Cell-cycle analysis of SMMC-7721 cells after treatment with 500 μg/ml Arg–$GlcNH_2$ for 96 h (%)

Group	G0/G1	S	G2/M
Control	76.78 ± 2.31	18.26 ± 0.86	4.96 ± 1.12
Arg–$GlcNH_2$	58.23 ± 0.65	36.63 ± 2.15	2.67 ± 1.03

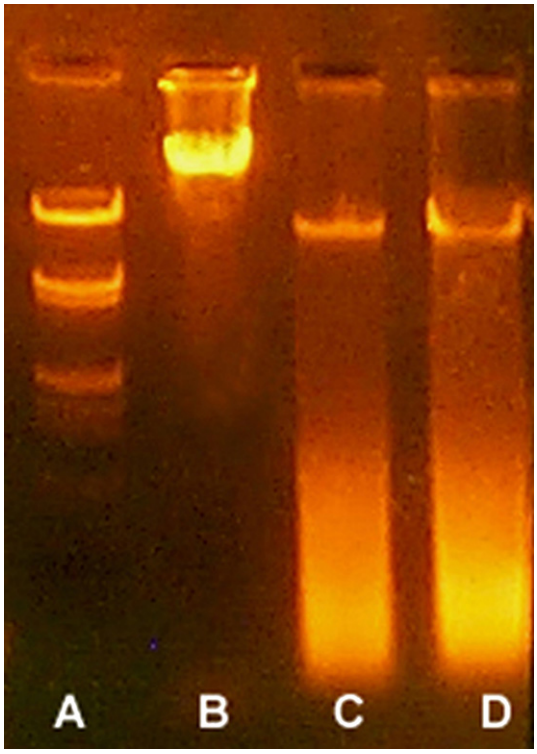


Fig. 6. Agarose gel electrophoresis of DNA extracted from control cells and cells treated with Arg–$GlcNH_2$ for 96 h. (A) DNA marker; (B) Control; (C) 200 μg/ml; (D) 500 μg/ml.

3.4. Effect of Arg–$GlcNH_2$ on DNA fragmentation of SMMC-7721 cells

Agarose gel electrophoresis of DNA was performed, as DNA fragmentation into oligonucleosome-length fragments was one commonly used marker for cells undergoing apoptosis (Thompson, 1995). It was found that SMMC-7721 cells underwent apoptosis after treatment with 200 and 500 μg/ml Arg–$GlcNH_2$ for 96 h, respectively (Fig. 6). Our result was consistent with observations made by other investigators (Kosmider et al., 2004; Sakahira, Enari, Ohsawa, Uchiyama, & Nagata, 1999).

4. Conclusion

A new *O*-carboxymethyl glucosamine was prepared in alkaline ethanol, and the carboxymethyl degree was 97.45%. Then a new amino acid sugar conjugate, arginine–glucosamine was synthesized and characterized. The new compound could lead to a concentration-dependent reduction in human hepatoma cell SMMC-7721 growth. The inhibition ratio was higher than that of D-glucosamine hydrochloride at the same concentration. The manner of arginine–glucosamine cytocidal activity was concluded to be apoptosis as assayed by DNA agarose gel electrophoresis and flow cytometry.

Acknowledgements

This work was supported by the Hi-Tech Research and Development Program (863) of China (No. 2001AA625050). The authors are grateful to Prof. Mingkun Sun for his critical remarks on this manuscript. Thanks are also given to Miss Xiuli Zhang for her kind help with the NMR analysis.

References

Akiyama, K., Kawazu, K., & Kobayashi, A. (1995). A novel method for chemo-enzymatic synthesis of elicitor-active chitosan oligomers and partially *N*-deacetylated chitin oligomers using *N*-acylated chitotrioses as substrates in a lysozyme-catalyzed transglycosylation reaction system. *Carbohydrate Research, 279*, 151.

Chen, L. Y., Du, Y. M., & Liu, Y. (2000). Structure-antimicrobial ability relationship of carboxymethyl chitosan. *Journal of Wuhan University, 46*(2), 191.

Chen, X. G., Wang, Z., Liu, W. S., & Park, H. J. (2002). The effect of carboxymethyl-chitosan on proliferation and collagen secretion of normal and keloid skin fibroblasts. *Biomaterials, 23*(23), 4609.

Chen, Q., Yang, F., & Du, Y. G. (2005). Synthesis of a C-3-symmetric $(1 \rightarrow 6)$-*N*-acetyl-*β*-D-glucosamine octadecasaccharide using click chemistry. *Carbohydrate Research, 340*(16), 2476.

Friedman, S. J., & Skehan, P. (1980). Membrane-active drugs potentiate the killing of tumor cell by D-glucosamine. *Proceedings of the National Academy of Sciences of the USA, 77*(2), 1172.

Ingle, T. R., Vaidya, S. H., & Pai, M. U. (1973). Production of D-glucosamine hydrochloride (GAH) from fish canning waste. *Research and Industry, 18*(2), 54.

Jin, D. M., & Zhu, W. X. (Eds.). (1996). *Research method of coordination chemistry*. Beijing: Science Publication Press.

Kosmider, B., Osiecka, R., Ciesielska, E., Szmigiero, L., Zyner, E., & Ochocki, J. (2004). Induction of apoptosis and necrosis in lymphocytes by the *cis*-Pt(II) complex of 3-aminoflavone in comparison with *cis*-DDP. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 558*(1–2), 169.

Lin, P. S., Ho, K. C., & Yang, S. J. (1996). Tirapazamine (SR 4233) interrupts cell-cycle progression and induces apoptosis. *Cancer Letters, 105*(2), 249.

Martin, G. G., Castro, C., Moy, N., & Rubin, N. (2003). *N*-acetyl-D-glucosamine in crustacean hemocytes; possible functions and usefulness in hemocyte classification. *Invertebrate Biology, 122*(3), 265.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods, 65*(1–2), 55.

Murata, M., Nabeshima, S., Kikuchi, K., Yamaji, K., Furusyo, N., & Hayashi, J. (2006). A comparison of the antitumor effects of interferon-α and β on human hepatocellular carcinoma cell lines. *Cytokine, 33*(3), 121.

Muzzarelli, R. A. A. (1988). Carboxymethylated chitins and chitosans. *Carbohydrate Polymers, 8*(1), 1.

Muzzarelli, R. A. A., Tanfani, F., Emanuelli, M., & Mariotti, S. (1982). *N*-(carboxymethylidene)chitosans and *N*-(carboxymethyl)chitosans: novel chelating polyampholytes obtained from chitosan glyoxylate. *Carbohydrate Research, 107*(2), 199.

Ning, Y. C. (Ed.). (2000). *Structural identification of organic compounds and organic spectroscopy* (2nd edition). Beijing: Science Publication Press.

Nishimura, S., Nishi, N., & Tokura, S. (1986). Bioactive chitin derivatives. Activation of mouse-peritoneal marcrophages by *O*-(carboxymethyl) chitins. *Carbohydrate Research, 146*(2), 251.

Park, K. G., Hayes, P. D., Garlick, P. J., Sewell, H., & Eremin, O. (1991). Stimulation of lymphocyte natural cytotoxicity by L-arginine. *Lancet, 337*(8742), 645.

Peng, Y. F., Han, B. Q., Liu, W. S., & Xu, X. J. (2005). Preparation and antimicrobial activity of hydroxypropyl chitosan. *Carbohydrate Research, 340*(11), 1846.

Qin, X. Q., Runkel, L., Deck, C., DeDios, C., & Barsoum, J. (1997). Interferon-beta induces S phase accumulation selectively in human transformed cells. *Journal of Interferon and Cytokine Research, 17*(6), 355.

Reynolds, J. V., Daly, J. M., Shou, J., Sigal, R., Ziegler, M. M., & Naji, A. (1990). Immunologic effects of arginine supplementation in tumor-bearing and non-tumor-bearing hosts. *Annals of Surgery, 211*(2), 202.

Rinaudo, M., Dung, P. L., Gey, C., & Milas, M. (1992). Substituent distribution on *O*,*N*-carboxymethylchitosans by ^{1}H and ^{13}C n.m.r. *International Journal of Biological Macromolecules, 14*(3), 122.

Sakahira, H., Enari, M., Ohsawa, Y., Uchiyama, Y., & Nagata, S. (1999). Apoptotic nuclear morphological change without DNA fragmentation. *Current Biology, 9*(10), 543.

Sambrook, J., & Russell, D. (Eds.). (2001). *Molecular cloning: A laboratry manual*. New York: Cold Spring Harbor Lab (CSHL) Press.

Shigemasa, Y., Matsuura, H., Sashiwa, H., & Saimoto, H. (1996). Evaluation of different absorbance ratios from infrared spectroscopy for analyzing the degree of deacetylation in chitin. *International Journal of Biological Macromolecules, 18*(3), 237.

Thompson, C. B. (1995). Apoptosis in the pathogenesis and treatment of disease. *Science, 267*(5203), 1456.

Wang, S. H., & Chen, J. C. (2005). The protective effect of chitin and chitosan against vibrio alginolyticus in white shrimp *Litopenaeus vannamei*. *Fish & Shellfish Immunology, 19*(3), 191.

Wang, Z., Qiao, Y., Huang, G. S., Wang, A. Q., Zhang, Y. Q., Feng, J. L., et al. (2003a). Glucosamine and glucosamine hydrochloride induced leukemia cell line K562 differentiation into macrophage. *Chinese Pharmacological Bulletin, 19*(3), 290.

Wang, Z., Qiao, Y., Huang, G. S., Wang, A. Q., Zhang, Y. Q., Feng, J. L., et al. (2003b). Induction of macrophagic differentiation of leukemia cell line K562 by *N*-acetyl-D-glucosamine. *Journal of the Fourth Military Medical University, 24*(1), 46.

Wang, Q., & Wang, A. Q. (2004). Study on the synthesis and properties of *N*-Succinyl-hitosan. *Journal of Functional Polymers, 17*(11), 51.

Yang, J. H., Du, Y. M., & Qin, C. Q. (2003). Applications of infrared spectroscopy and nuclear magnetic resonance spectroscopy in the studies of the structure of chitin and chitosan. *Journal of Analytical Science, 19*(3), 282.

Zhang, L., Liu, W. S., Han, B. Q., Peng, Y. F., & Wang, D. F. (2006). Antitumor activities of D-glucosamine and its derivatives. *Journal of Zhejiang University SCIENCE B, 7*(8), 608.

Zhao, Z. P., Wang, Z., Ye, N., & Wang, S. C. (2002). A novel *N*,*O*-carboxymethyl amphoteric chitosan/poly(ethersulfone) composite MF membrane and its charged characteristics. *Desalination, 144*(1-3), 35.

Zheng, Y. N., Matsuura, Y., Han, L. K., Takaku, T., Xiang, L., Kameda, K., et al. (1996). Isolation and structure elucidation of a new amino acid derivative from red ginseng. *Acta Pharmaceutica Sinica, 31*(3), 191.

Zheng, Y. N., Zhang, J., & Okuda, H. (1998). Effect of arginine derivatives on microcirculation. *Journal of Jilin Agricultural University, 20*(4), 45.