CIENCIA 21(2), 103 - 107, 2013
Maracaibo, Venezuela

2-N-Acetylglucosamine-α-O-threonine, an amino acid derivative from the marine sponge *Cinachyrella kuekenthali*

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Received: 10-10-12 Accepted: 12-03-13

Abstract

A new amino acid derivative, 2-*N*-acetylglucosamine-α-*O*-threonine [2-*N*-AcNGlc-α-*O*-Thr, (**1**)], was isolated from the marine sponge *Cinachyrella kuekenthali*. The structure assignment is based on interpretation of NMR spectroscopic data. The orientation of the α-O-glucosamine bond was deduced from the value of the (J = 3.7 Hz) coupling constant of the anomeric proton and the position of the 2-*N*-acetylglucosamine moiety on the value of the (δ_C 56.2) carbon chemical shift of the C2-position. The amino acid derivative was inactive on the Sk Br-3 (breast) and HT-29 (colon) human cancer cell lines and MA-104 kidney monkey cell line.

Keywords: *Cinachyrella kuekenthali*, Amino acid, 2-*N*-acetylglucosamine-α-*O*-threonine.

2-*N*-acetilglucosamina-α-*O*-threonina, un aminoácido derivado de la esponja marina *Cinachyrella kuekenthali*

Resumen

Un nuevo aminoácido derivado de treonina, 2-*N*-acetilglucosamina-α-*O*-treonina [2-N-AcNGlc-α-*O*-Thr, (**1**)], fue aislado de la esponja marina *Cinachyrella kuekenthali*. La asignación estructural es en base a la interpretación de datos espectroscópicos de RMN. La orientación del enlace α-O-glucosamina fue deducida del valor de la constante de acoplamiento del protón anomérico (J = 3,7 Hz) y la posición del residuo 2-*N*-acetilglucosamina fue establecida por el desplazamiento químico (δ_C 56,2) del carbono en la posición C-2 del anillo glicosídico. El derivado del aminoácido treonina fue inactivo contra las líneas celulares de cáncer humano (mama) Sk Br-3, (colon) HT-29 y la línea celular de riñón de mono MA-104.

Palabras clave: *Cinachyrella kuekenthali*, aminoácido, 2-*N*-acetilglucosamina-α-*O*-treonina.

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Introduction

The *Cinachyrella* genus has received only scarce attention from the chemical point of view. Previously reported compounds present unusual chemical structures [1-4]. Fatty acid composition of the phospholipids from *Cinachyrella* species has been reported [5-8]. The most important active compound from this genus is the highly cytotoxic macrolide cinachyrolide A [3]. Extracts of *C. kuekenthali* exhibit antimicrobial effects in bacteria and fungi [9]. In previous research at our laboratory we found that components in the polar extracts of this sponge have potent toxic effects in mice and cytotoxic in cell lines [10]. Moreover, we reported that a new poly-1,3-propanediamine type long-chain polyamines baptized as cinachyramides isolated from *Cinachyrella kuekenthali* showed cytotoxicity with LC_{50} values of 3.07 and 3.56 μg/mL against the HT-29 (colon) and Sk Br-3 (breast) human cancer cell lines, respectively [11]. The cinachyramides showed antibacterial activity more pronounced against Gram positive bacteria than Gram negative, with LC_{50} values determined as 2.58, 5.91, 16.40 and 56.40 μg/mL against *B. subtilis, M. luteus, E. faecalis* and *E. coli,* respectively, causing morphologic changes in *B. subtillis* and *E. coli* observed by MEB [12].

By guided mutagenesis 31 unnatural amino acids were incorporated in the metabolism of *Escherichia coli* [13]. Among the unnatural amino acids are *N*-acetylglucosamine-β-*O*-serine (AcNGlc-β-O-Ser) and *N*-acetylgalactosamine-α-*O*-threonine (AcNGlc-α-O-Thr) [13, 14]. We now wish to report the isolation and structural characterization of 2-*N*-acetylglucosamine-α-*O*-threonine (2-*N*-AcNGlc-α-*O*-Thr, 1) from *Cinachyrella kuekenthali,* based on interpretation of NMR spectroscopic data. This amino acid derivative has not previously been reported from natural sources, but is present in synthetic mucin repeats [15]. Moreover, is make by O-linked between *N*-acetylglucosamine-threonine 32 on histone H3 as a regulator of mitosis-specific phosphorilations and as a posttranslational modification consisting of a single *N*-acetylglucosamine moiety attached by an *O*-β-glycosidic linkage to serine and threonine residues of both nuclear and cytosolic [16, 17].

Materials and methods

Cinachyrella kuekenthali (Uliczka, 1929) was collected in Mochima Bay (Taguapire Inlet) Venezuela in June 2003. Identification of the sponge was done by Maria Elena Amaro MSc of the Marine Bioactivity Laboratory, Instituto Oceanográfico de Venezuela, Universidad de Oriente; and a voucher specimen is preserved under the code IOV-PDT-2111-J.

The fresh sponge (837g) liquefied with 0.8 L technical grade ethanol generates a concentrated aqueous solution after removing the ethanol by rotaevaporation and therefore bipartitioned with acetone. The aqueous phase after lyophilized was purified by molecular exclusion chromatography (3 cm Ø × 78 cm) on Bio-Gel® P2 (Bio-Rad, 100-200 mesh) using acetic acid 1M as mobile phase. Fractions containing 200 drops of eluate were collected and combined in fractions generated by plotting the value of the absorbance at 250 nm against the normalized volume. The second fraction (BGP II) was separated in three fractions on G 15 Sephadex gel using distilled water as mobile phase. The Sephadex G II fraction was purified by HPLC through a semi-preparative molecular exclusion column, Protein Pak 60 (particle size 100 μm; 7.8 mm Ø × 300 mm, Waters) isocratically with H_2O/AcCN/2-propanol (3:5:2) as mobile phase with a flow of 0.5 mL/min and monitored by the absorbance at 230 and 265 nm. The peak appearing at 21.28 min was repurified by HPLC on a semi-preparative reverse phase column, Protein & Peptide C18 (particle size 10 μm; 10 mm Ø × 250 mm, 218TP1010 Vydac) using a linear gradient H_2O(0.12% TFA)/ACN(0.10% TFA) 30% during 30 min as mobile phase with a flow of 1 mL/min and monitored by absor-

bance at 230 nm, a peak with a retention time of 26.28 min.

2-acetamido-2-deoxy-α-glucopyranosyl-1-3-threonine or 2N-acetylglucosamine-α-*O*-threonine (2N-AcNGlc-α-*O*-Thr)

^{1}H NMR (400 MHz DMSO-d_6) δ (ppm). See Table 1. The coupling constants for J_{NH} for the ammonium group appearing at 7.13 ppm (3H, dt, $^{+}NH_3$) was determined as $J^{14}_{N\text{-}H}$ = 51.1 Hz and as $J^{14}_{N\text{-}C\text{-}H}$ = 9.3 Hz are characteristic values for $^{+}NH_4$ type ion. ^{13}C NMR (100 MHz DMSO-d_6). See Table 1. The carbon signal appearing at 68.8 ppm display a coupling, $J_{C\text{-}}{}^{14}{}_{N}$ = 15.3 Hz, supporting the assignment of this carbon to the threonine C_α.

Results and discussions

An ethanolic extract of freshly collected *Cinachyrella kuekenthali* gave, after repeated chromatographic separations, rise to the isolation of a strongly hydrophilic compound, which was subjected to extensive NMR investigations.

The presence of a threonine moiety reveals itself from ^{1}H and ^{13}C NMR data corresponding to a carboxylic acid group (12-13, 171.8 ppm) connected to the C_α-H group (4.33, 68.8 ppm), which is in turn connected to the – $^{+}NH_3$ group (7.13 ppm, dt, $J^{14}_{N\text{-}H}$ = 51.1 Hz and $J^{14}_{N\text{-}C\text{-}H}$ = 9.3 Hz) and the C_β-H (4.17, 74.0 ppm) group. The threonine γ-methyl group (1.14, 18.9 ppm) concludes the threonine moiety connected further through the hydroxyl function.

The 2-acetamido-2-deoxyglucose unit was identified from NMR data and could be distinguished from the 2*N*-acetylgalactosamine-α-*O*-threonine, (C-2 ~ 50 ppm), [18-20] by the value of the C-2 signal (56.2 ppm). The acetyl group (1.92, 22.5 ppm) is connected to an –NH group (8.04 ppm, exchangeable with D_2O), which is in turn connected to the 2-CH group of the deoxyglucose. The 2-C-group is attached to the anomeric –CH (4.76, 100.2 ppm) displaying the α configuration and connected through oxygen to the β-position of the threonine moiety as evidenced from a HMBC connection between the anomeric proton and the threonine

Table 1
NMR data for 2-*N*-acetylglucosamine-α-*O*-threonine (2-*N*-AcNGlc-α-*O*-Thr) (1) in DMSO-d_6

δ_H ppm (mult, *J*=Hz)	COSY H-H	HSQC (δ_C ppm)	HMBC (δ_C ppm)
1.14 (d, $J_{\beta CH,\gamma CH}$=6.2 Hz, 3H, ThrγCH_3)	4.17	18.9	56.2; 74.0
1.92 (s, 3H, CH_3CONH-)		22.5	169.7
3.47 (m)	3.69; 4.76	60.6; 68.9; 69.8	68.9; 69.8; 71.7
3,69 (m)	3.47	68.9, 71.7	60.6; 68.9; 100.2
4.17 (m, *J*=2.9; 6.3 Hz, 1H, ThrβCH)	1.14	74.0	100.2
4.33 (m, *J*=3.0; 6.0 Hz, 1H, H-2)	8.04	56.2	171.8
4.76 (d, *J*=3.7 Hz, 1H, H-1)	3.47	100.2	69.8; 71.7; 74.0
7.13 (dt, *J*=9.3, 51.1 Hz, 3H, H_3N^{+}-C-COOH)			
8.04 (d, *J*=8.8 Hz, 1H, AcNH)	4.33		169.7
	169.7		
	171.8		
12-13 (1H, -COOH)			

δ_C (ppm) assignments are based on ^{13}C NMR experiment. NMR data were measured in DMSO-d_6 at 400 MHz for ^{1}H and H-H COSY, 100 MHz for ^{13}C, HSQC (*J* = 120 Hz) and HMBC (*J* = 7 Hz).

β-carbon. The α orientation assignment is based on the value of the coupling constant, $^3J_{ac}$ 3.7 Hz, between the anomeric proton and the axial proton at C-2 in the pyranose ring (characteristic value $^3J_{ac} \approx 4$ Hz) as opposed to a characteristic value for $^3J_{ac} \approx 9$ Hz in a pyranose β configuration [20].

The connectivity between the 2-deoxyglucose ring and the threonine moiety was confirmed by the long-range C-H correlation between the anomeric carbon signal (100.2 ppm) and the threonine β-proton signal C_β-H 4.17 ppm). This was strengthened by the long-range correlation between the anomeric proton signal (4.76 ppm, $^3J_{ac}$ = 3.7 Hz) and the threonine β-carbon signal (C_β 74.0 ppm), which in turn were coupled to the threonine γ-methyl group proton (C_γ-H_3 1.14 ppm, J = 6.2 Hz). The presence of the acetamido moiety was confirmed by the long-range C-H correlation between the –NH group proton signal (δ_H 8.04) and the carbonyl carbon signal (δ_C 169.7), which is in turn coupled to the methyl group proton (δ_H 1.92), consistent with a amide functionality. The connectivity between the acetamido moiety and the 2-deoxyglucose ring was confirmed by H-H correlation in the COSY spectrum, the coupling between the anomeric proton signal (δ_H 4.76) and the –CH methine group proton signal (4.33, 56.2 ppm) of the glucose ring, which is consistent with the connectivity between acetamido moiety and deoxyglucose ring at C-2 position by the chemical shift value of the C-2 [18-20].

Because of lack of material the absolute configurations could not be determined, but we suggest that the derivative **1**, in analogy with related compounds, to be composed of D-threonine and L glucose.

Cytotoxicity

The cytotoxic activity was determined against colon (HT-29) and breast (Sk Br-3) human cancer cell lines and kidney monkey (MA-104) normal cell line according to the method of Mosmann [21]. The amino acid (**1**) was assayed at 0.3 mg/mL or below.

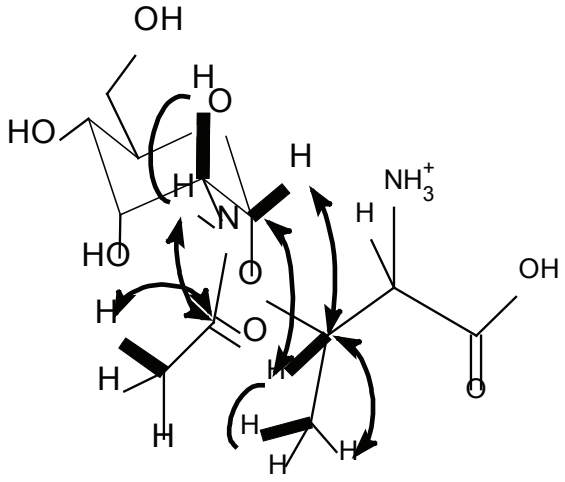

Figure 1. Structure of 2-*N*-acetylglucosamine-α-*O*-threonine (2-*N*-AcNGlc-α-*O*-Thr) (**1**). Selected correlations, COSY H-H (half circle lines without arrowheads), HSQC (fat bonds) and HMBC (half circle lines with double arrowhead).

Conclusions

The 2-acetamido-2-deoxyglucose unit was identified from NMR data by the value of the C-2 signal (56.2 ppm). The connectivity between the acetamido moiety and the 2-deoxyglucose ring was confirmed by the coupling between the anomeric proton signal (δ_H 4.76) and the –CH methine group proton signal (4.33, 56.2 ppm) of the glucose ring, which is consistent with the connectivity between acetamido moiety and deoxyglucose ring at C-2 position by the chemical shift value of the C-2. The α configuration and connectivity through oxygen to the β-position of the threonine moiety is evidenced from the connection between the anomeric proton (4.76, 100.2 ppm) and the threonine β-carbon (74.0 ppm). The α orientation assignment is based on the value of the coupling constant, $^3J_{ac}$ 3.7 Hz, between the anomeric proton and the axial proton at C-2 in the py-

ranose ring (characteristic value $^3J_{ac} \approx 4$ Hz) as opposed to a characteristic value for $^3J_{ac} \approx 9$ Hz in a pyranose β configuration.

Acknowledgments

The authors are grateful to Human Resources, Universidad de Oriente, Venezuela, for financing studies at IVIC (WH), to the Lic. Y. Higuerey, CBB, IVIC, Venezuela, for technical support with cell cultures, and M.E. Amaro MSc. Oceanographic Institute of Venezuela, Universidad de Oriente, for the identification of the sponge.

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