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SYNTHESIS OF ASSIGNED STRUCTURE OF CAIROMYCIN A, 6-ISOPROPYL-2,5-DIKETOPIPERAZINE-3-ACETIC ACID AND ITS B-LACTAM ANALOGUE N`- (3-METHYL-1-HYDROXYBUTYR-2-YL) AZETIDIN-2-ONE-4-CARBOXIMIDE

Faiq H. S. Hussain*

Dept. of Medical Analysis, Faculty of Science, Tishk International University, Erbil, Kurdistan Region-Iraq (faiq.hussain@ishik.edu.iq)

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ABSTRACT:

In the present investigation, the research carried out into the total synthesis of the assigned structure of cairomycin A, 6-isopropyl-2,5-diketopiperazine-3-acetic acid from its precursors L-valine and L-aspartic acid, and its analogue N`-(3-methyl-1-hydroxybutyr-2-yl)azetidin-2-one-4-carboximide from 4-vinylazetidi-2-one through two different sequence reactions. Structures of the synthesized products were identified by using chemical and physical methods such as elemental analysis, IR, ^{1}H-NMR and MS. The obtained results were compared with those reported, it was found that neither of the synthesized compounds was Cairomycin A.

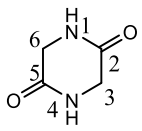
KEYWORDS: Cairomycin A, 2,5-Diketonpiperazine, β-Lactam, Azetidine, Antimicrobial.

1. INTRODUCTION

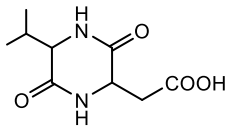
2,5-Diketonpiperazine (1), is a smallest class of cyclopeptide, which produced from the condensation and cyclization of two amino acids (Dinsmore *et al* 2002). Diketopiperazine and its derivatives are known to be associated with broad spectrum of biological activity like antitumor (Nicholson *et al* 2006), antiviral (Sinha *et al* 2004), antifungal (Houston *et al* 2004), and antibacterial (Abraham 2005).

A large number of these compounds occur in natural products of polypeptides and have been isolated from plants, animals, bacteria and fungi (Borthwick 2012), but may be easily synthesized (Delaforge *et al* 2001). However, many synthetic methods are available mainly based on the dipeptide reactions by conventional methodology from α-amino acids (Fischer 2003; O'Neill *et al* 2007; Sun 2014).

Shimi and Fathey isolated a cyclic antibiotic from the fermentation of *Streptomyces sp*. strain As-C-19 which obtained from the soil of Cairo (Shimi and Fathey 1981), this antibiotic was designated as 6-isopropyl-2,5-diketopiperazine-3-acetic acid, cairomycin A (2) which is peptide with potent activity against Gram positive bacteria. Acid hydrolysis of this isolated diketopiperizene afforded two amino acids, L-valine and L-aspartic acid.



(1)



(2)

The designed structure (2) was prepared by reported literature procedure from t-butyloxycarbonyl L-valin and L-aspartic acid dibenzyl ester-p-toluene sulfonate (Kumar A. *et al* 1985).

Here, we propose our design strategy to record the total synthesis of compound (2) from its precursors L-aspartic acid and L-valine. So, the aim is to follow a step by step tuning of the diketopeprizine structure on the one hand, and compare its spectroscopic data, physical properties with those reported, on the other hand, in order to get the required information on the structure for the desired compound, with the help of suitable and corroborating biological evaluations.

2. EXPERIMENTAL

2.1 Reagents and Techniques:

Melting points were determined using Gallenkamp melting point apparatus and uncorrected. IR spectra were obtained using a Perkin-Elmer 197 instrument. ^{1}H-NMR spectra were recorded using Perkin-Elmer R34 (220 MHz) instrument with (TMS) as a standard. Mass spectra were determined using Kratos MS45 instrument. Most of solvents were distilled prior to use. All fine chemicals were purchased from Sigma-Aldrich and used without any further purification.

2.2 Synthetic Procedures:

Preparation of carbobenzoxy valine (4)

To a solution of L-valine (5.85 g, 50 mmol) in an aqueous solution of sodium hydroxide (12.6 ml, 4N) at 0ºC were added concurrently during 1 h carbobenzoxy chloride (10.7 ml, 12.78 mmol) and sodium hydroxide solution (16.6 ml, 4N) with vigorous stirring.

The resulting solution was acidified with hydrochloric acid (1M) and the viscous solution that formed was extracted with vigorous stirring into ethyl acetate (4x100 ml). The combined extracts were cooled to 0ºC and extracted with ice-cold 10% aqueous sodium carbonate solution (3x100 ml). The oily product which separated upon acidification of the combined sodium carbonate extracts solidified on standing overnight in the cold. The obtained product was filtered off, washed with cold water and dried (over P_2O_5) in vacuo to afford the pure carbobenzoxy valine (10.3 g, 82 %) as a white solid; m.p: 54-55 ºC. I.R (cm^{-1}): 3400, 3200-2750, 1740, 1650. ^{1}H-NMR ($CDCl_3$): δ= 0.9 (3H, d, J 4.0 Hz, CH_3); 1.05 (3H, d, J 4.0 Hz, CH_3): 2.2 (1H, m, C<u>H</u>$(CH_3)_2$); 4.3 (1H, m, $NCHCO_2$); 5.15 (2H, s, OCH_2); 5.5 (1H, m, NH); 7.4 (5H, s, Ph); 10.6 (1H, s, OH). MS: m/z: 251 (M^+), 206 (M^+-CO_2H), 162 ($PhCH_2OCO$-$N{=}C^+H$), 108 ($PhCH_2OH$), 91 ($PhCH_2^+$). Anal.

calc. for $C_{13}H_{17}NO_4$: C = 62.2, H = 6.8, N = 5.5%, found: C = 62.1, H = 7.0, N = 5.5%

Preparation of succinimido N-(N-carboxy-L-valyl)oxy-benzyl ester (5)

To an ice-bath cooled mixture of carbobenzoxy valine (10.04 g, 40 mmol) and N-hydroxysuccin-imide (4.6 g, 40 mmol) in dioxane (10 ml) was added N,N'-dicyclohexylcarbodiimide (8.24 g, 40 mmol). A white precipitate formed immediately and the mixture was allowed to stand overnight in a refrigerator (approximately -10°C). The dicyclohexylurea was filtered off and washed with dioxane (10 ml); when the filtrate was concentrated and the residue was triturated with ether, a white solid crystallized out on cooling. Recrystallization of crude product from isopropanol gave the desired product (11.2 g, 80 %) as a white crystalline solid; m.p.: 114-115°C. I.R (cm^{-1}): 3350, 1815, 1780, 1720. ^{1}H-NMR ($(CD_3)_2CO$): δ= 1.05 (6H, d, J = 6.0 Hz, 2 CH_3); 2.3 (1H, m, $CH(CH_3)_2$); 2.8 (4H, s, $2CH_2$); 4.5 (1H, m, $NCHCO_2$); 5.05 (2H, s, OCH_2); 6.9 (1H, br., NH); 7.3 (5H, s, Ph). MS: m/z; 348 (M^+), 206 [$(CH_3)_2CH$-C^+H-$NHCO_2CH_2Ph$), 162 ($PhCH_2OCO$-N=C^+H), 115 (hydroxysuccinimide); 91 ($PhCH_2$). Anal. calc. for $C_{17}H_{20}N_2O_6$: C = 58.6, H = 5.7, N = 8.0%, found: C = 58.5; H = 5.9; N = 8.0%

Preparation of L-aspartic acid dimethyl ester hydrochloride (7)

Thionyl chloride (20 ml, 32.62 g, 270 mmol) was added carefully with stirring for 30 min. to methanol (100 ml) at room temperature (the reaction was very exothermic). To the mixture L-aspartic acid (20 g. 150 mmol) was added with continues stirring for 60 h after which refluxed for 30 min. To the cooled mixture dry ether was added until a white precipitated stopped forming with further cooling. The product was filtered off, washed with cold ether and dried in vacuo over P_2O_5 , yielding the product as a white solid (18.7 g, 63%); m.p: 113-114°C. I.R (cm^{-1}): 3700-2800, 1680, 1600 cm^{-1}. ^{1}H-NMR ($CDCI_3$): δ= 3.05 (2H, d, J = 6.0 Hz, CH_2CO_2); 3.7 (6H, s, 2 OCH_3); 4.3 (1H, t, J = 6.0 Hz, CH); 8.8 (3H, br., NH_3). MS: m/z: 198 (M^+), 102 [M^+-(MeOCO and HCl), 59 (MeOCO). Anal. calc. for $C_6H_{12}ClNO_4$: C = 36.4, H = 6.1, N = 7.1, Cl = 18.0 %, found: C = 36.2, H = 6.3, N = 7.1, Cl = 17.9 %.

Preparation of carbobenzoxy valine-methyl aspartate dipeptide (8)

To a solution of succinimido N-(N-carboxy-L-valyl) oxybenzyl ester (5) (7.0 g, 20 mmol) in dimethoxyethane (20 ml) were added dimethyl aspartate hydrochloride (239) (3.95 g, 20 mmol) and N- methylmorpholine (2.2 ml, 2.02 g, 20 mmol), and the mixture was stirred for 45 h at room temperature. The reaction mixture was poured into cold water (100 ml) and a white solid crystallized, which was filtered off, and washed with cold water. Recrystallization from ethanol-water afford dipeptide (240) as a white solid (7.2 g. 91%); m.p: 135-136 °C. I.R (cm^{-1}): 3300, 3260, 1750, 1722, 1682, 1648. ^{1}H-N.M.R. ($(CD)_3CO$): δ 0.94 (3H, d, J = 6.0 Hz, CH_3); 0.98 (3H, d, J= 6.0 Hz, CH_3); 2.15 [1H, m, C<u>H</u>$(CH_3)_2$); 2.87 (2H, d, J = 6.0 Hz, CH_2CO_2); 3.63 (3H, s, OCH_3); 3.68 (3H, s, OCH_3); 4.12 (1H, m, C<u>H</u>CH_2); 4.84 [1H, m, C<u>H</u>$CH(CH_3)_2$; 5.09 (2H, s, CH_2Ph); 6.35 (1H, br., NH); 7.38 (5H, s, Ph); 7.65 (1H, br., NH). MS: m/z; 394 (M^+), 234 (M^+-$MeO_2CCH(NH)CH_2CO_2Me$), 206 [$(CH_3)_2CHC^+HNHCO_2CH_2Ph$], 162 ($PhCH_2O_2CN$=$C^+$H), 91 ($PhC^+H_2$), 83 [$(CH_3)_2CHCH$ =C=O]. Anal. calc. for $C_{19}H_{26}N_2O_7$: C = 57.8, H = 6.6, N = 7.1 %, found: C = 57.8, H = 6.9, N = 7.1 %.

Preparation of 6-isopropyl-2,5-diketopiperazine-3-methyl acetate (10)

The dipeptide amino acid (9) (4.0 g, 10 mmol) was dissolved in 0.1 M acetic acid-butan-2-ol (200 ml) and 10% palladium on charcoal (0.2 g) was added. The mixture was stirred under an atmosphere of hydrogen for 2h after which it was filtered through a celite layer, and a white solid compound crystallized out of the clear colorless solution. The mixture was refluxed for 3h to ensure that cyclization had taken place. The white crystals which formed by concentrating the solution were filtered off and dried over P_2O_5.The desired cyclized compound (242) was obtained (2.13 g, 92%) as a white crystalline solid; m.p: 229-231 °C. I.R (cm^{-1}): 3200, 3050, 1750, 1670 (br.). ^{1}H-NMR (DMSO-d_6): δ = 0.84 (3H, d, J = 6.0 Hz, CH_3); 0. 95 (3H, d, J = 6.0 Hz, CH_3): 2.2 [1H, m, C<u>H</u>$(CH_3)_2$]: 2.66 and 2.71 (each 1H, d, J = 4.0 Hz, CH_2); 3.6 (3H, s, OCH_3); 3.78 (1H, m, C<u>H</u>CH_2) 4.24 [1H, m, C<u>H</u>$CH(CH_3)_2$], 8.04 (1H, s, NH); 8.09 (1H, s, NH). MS: m/z: 228 (M^+), 213 (M^+-CH_3), 197 (M^+-OCH_3), 112 (M^+-$CH(CH_3)_2$ and $CH_2CO_2CH_3$). Anal. calc. for $C_{10}H_{16}N_2O_4$: C= 52.6, H= 7.0, N= 12.3 %, found: C= 52.7, H= 6.8, N= 12.3 %.

Preparation of 6-isopropyl-2,5-diketopiperazine-3- acetic acid (2)

A solution of 6-isopropyl-2,5-diketopiperazine-3-methyl acetate (10) (1.14 g, 5 mmol) and 10% aqueous potassium hydroxide solution (2.6 ml) in methanol (12 ml) was warmed to obtain a clear solution which was stirred overnight at room temperature. The potassium salt was formed as a white solid compound. After the methanol was evaporated, the residue was dissolved in water (15 ml) and acidified with dilute hydrochloric acid. A white solid crystallized upon acidification obtained which was filtered off (0.8 g, 75%). An analytical pure sample was produced by sublimation (120-130°C, 0.5 mmHg); m.p: 222-224 °C. I.R (cm^{-1}): 3200, 3550-2200, 1725, 1670-1620 (br.). ^{1}H-NMR (DMSO-d_6): δ= 0.83 (3H, d, J = 6.0 Hz, CH_3); 0.91 (3H, d, J = 6.0 Hz, CH_3); 2.1 [1H, m, C<u>H</u>$(CH_3)_2$]; 2.65 and 2.7 (each 1H, d, J= 4.0 Hz, CH_2); 3.58 [1H, m, C<u>H</u>$CH(CH_3)_2$]; 4.11 (1H, m, C<u>H</u>CH_2); 8.04 (1H, s, NH); 8.12 (1H, s, NH). MS: m/z: 114 (M^+), 199 (M^+-CH_3), 172 [M^+-$CH(CH_3)_2$], 112 [M^+-$CH(CH_3)_2$ and CO_2H]. Anal. calc. for $C_9H_{14}N_2O_4$: C= 50.5, H= 6.5, N= 13.1%, found: C= 50.6, H= 6.6, N= 13.3%.

Preparation of 4-vinylazetidin-2-one (15) (Brennan *et al*, 1980)

Butadiene (10 ml, 120 mmol) was liquefied at -78 °C in pressure tube and diluted with dry ether (10 ml). To this solution anhydrous sodium carbonate (0.5 g) was added and followed by chlorosulphonylisocyanate (5.5 ml, 8.97 g, 60 mmol). The tube was allowed to come to room temperature and after 16h the golden colored solution was poured cautiously into a vigorously stirred mixture of sodium sulfite (40 g), potassium hydrogen phosphate (24 g) and ice (100 g) in water (100 ml). After stirring with ether (100 ml) for 30 minutes the organic 1ayer was separated and the aqueous layer was re-extracted with ether (3 x 100 ml). The bulked ether was dried ($MgSO_4$) and concentrated in vacuo to yield the product as a pale yellow liquid (5. 32 g, 87%). An analytical sample was obtained by distillation

(collected fraction boiling at 68-69 ºC at 0.4 mmHg); I.R (cm^{-1}): 3250, 1745, 1645. ^{1}H-NMR ($CDCl_3$): δ= 2.5-2.8 (1H, m, CH_2CO cis); 2.95-3.30 (1H, m, CH_2CO trans); 4.1 (1H, m, CHNH); 5.1-5.4 (2H, m, $CH=CH_2$); 5.75-6.15 (1H, m, $CH=CH_2$); 7.15 (1H, m, NH). MS: m/z: 97 (M^+), 54 ($CH_2=CHCH=CH_2$), 42 (CH=C=O). Anal. calc. for C_5H_7NO : C= 61.8, H= 7.3, N= 14.4%, found: C= 61.6, H= 7.6, N= 14.1%.

Oxidation of 4-Vinylazetidin-2-one (15)

A. With ozone-permanganate

1. **In methanol**
   4-vinylazetidin-2-one (1.0 g, 10.3 mmol) was dissolved in methanol (30 ml), cooled to -78 °C and ozonized until a blue coloration persisted. The solution was then flushed with nitrogen to discard the blue coloration after which the methanol was evaporated under reduced pressure (no heating). The white ozonide was quickly dissolved in glacial acetic acid (25 ml) and a solution of potassium permanganate (1.7 g, 11 mmol) in water (30 ml) was added dropwise carefully. The mixture was stirred for 1.5 h at room temperature, the black color was discharged by dropwise addition of 30 % hydrogen peroxide. The solvent was evaporated under reducing pressure (no heating) and the residue was dissolved in water (30 ml); acidified with 1M hydrochloric acid and extracted with ethyl acetate (50 ml). The organic phase was separated, dried (Na_2SO_4) and evaporated. No characterizable product was obtained.

2. **In dichloromethane**
   The procedure was similar to the one used above, no required product could be isolated.

B. With ozone-pyridinium dichromate (Corey and Schmidt, 1979)

The prepared ozonide, as described above, was dissolved in DMF (7 ml), pyridinium dichromate (3.5 equivalent) was added and the mixture was stirred for 8 h at room temperature. The reaction mixture was poured into water (20 ml) and extracted with ethyl acetate (2 x 15 ml). The combined organic extracts were dried ($MgSO_4$) and evaporated. No oxidized product was obtained.

C. With periodate-permanganate (Lemieux and Rudloff, 1955)

To a solution of potassium carbonate (1.84 g, 13 mmol), potassium permanganate (0.14 g, 0.89 mmol) and sodium meta-periodate (11.4 g 53.3 mmol) in water (30 ml), was added 4-vinyl azetidin-2-one (245) (0.6 g, 6.6 mmol) and the mixture was stirred at room temperature for 10 h under nitrogen atmosphere. The mixture was extracted with ethyl acetate (4 x 25 ml), the combined organic extract was dried ($MgSO_4$) and evaporated in vacuo. No desired product was obtained.

Preparation of 1-(t-butyldimethylsilyl)-4-vinylazetidin-2-one (17)

To a solution of 4-vinylazetidin-2-one (4.0 g, 41 mmol) in DMF (50 ml) at 0°C was added triethylamine (6.34 ml, 4.6 g, 45 mmol), followed by t-butyldimethylsilyl chloride (6.75 g, 45 mmol) and the mixture was stirred for 1 h. The reaction mixture was poured into ether (100 ml) and extracted with water (5 x 100 ml) followed by brine (30 ml). The organic layer was separated, dried over $MgSO_4$ and concentrated under reduced pressure to give the product as a straw-colored liquid (7.75 g, 89%); I.R (cm^{-1}): 1750, 1290, 845. ^{1}H-NMR ($CDCl_3$): δ= 0.17 (3H, s, CH_3); 0.20 (3H, s, CH_3); 0,95 (9H, s, t-butyl); 2.55-2.90 (1H, dd, J = 3 Hz, $COCH_2$ trans); 3.10-3.50 (1H, dd, J = 6, 16 Hz, $COCH_2$ cis); 4.0 (1H, m, NCH); 5.0-6.2 (3H, m, vinyl). MS: m/z: 211 (M^+), 154 (M^+-Bu^t), 112 (M^+-Bu^t and $CH_2=C=O$). High Resolution MS for $C_{11}H_{21}NSiO$ M^+ = 211.1398, found M^+ = 211.1395.

Preparation of 1-(t-butyldimethylsilyl)-4-hydroxycarbonyl-azetidin-2-one (18)

Powdered potassium permanganate (6.0 g, 38 mmol) was added over 2 h to an acetone solution (25 ml) of 1-t-butyl-4-vinylazetidin-2-one (2.11 g, 10 mmol) containing powdered sodium bicarbonate (0.35 g) at 7 °C and the mixture was vigorously stirred for 4 h. Water (20 ml) was added to the residue after evaporating off the acetone. The solution was acidified with 1 M sulfuric acid and solid sodium bisulfite (5.0 g) was added. The resulting clear solution was extracted with ethyl acetate (3 x 50 ml) after which the organic phase was separated, dried ($MgSO_4$) and evaporated in vacuo. Recrystallization of the residue from toluene provided the pure product (1.95 g, 85 %) as a white solid; m.p: 130-131 °C. I.R. (cm^{-1}): 3600-2300, 1740, 1680. ^{1}H-NMR ($CDCl_3$): δ = 0.16 (3H, s, $SiCH_3$); 0.3 (3H, s, $SiCH_3$); 0.95 (9H, s, t-butyl); 3.12 (1H, dd, J= 2.8, 15 Hz, $COCH_2$ trans): 3.4 (1H, dd, J = 5.7, 15 Hz, $COCH_2$ cis); 4.09 (1H, dd, J = 2.8, 5.7 Hz, NCH); 8.37 (1H, br., OH). MS: m/z: 229 (M^+), 189 (HO_2CCH_2=NH-S+) 172 (M^+-t-butyl), 157 (M^+ $CH_2=CHCO_2H$). Anal. calc. for $C_{10}H_{19}NO_3Si$: C = 52.4, H = 8.4, N = 6.1, Si = 12.3 %, found: C = 52.5, H = 8.4, N = 6.2, Si = 12.0 %.

Preparation of 1-(t-butyldimethylsilyl)-4-(succinimido-N-oxycarbonyl) azetidin-2-one (19)

N,N-Dicyclohexylcarbodiimide (1.66 g, 8 mmol) was added to an ice-bath cooled solution of 1-(t-butyldimethylsilyl)-4-hydroxy carbonylazetidin-2-one (18) (1.83 g, 8 mmol) and N-hydroxy-succinimide (0.92 g, 8 mmol) in dry dioxane (4 ml). The mixture was allowed to stand overnight in the refrigerator (approximately -10°C) after which dicyclohexylurea was filtered off and washed with dioxane (4 ml). The clear filtrate was concentrated in vacuo to a colorless oil which crystallized on trituration under petroleum ether (b.p. 40-60°C) with cooling to yield the product as a white crystalline solid. Recrystallization from isopropanol gave the pure desired product (2.52 g, 97 %); m.p: 86-88°C. I.R (cm^{-1}): 1785, 1765, 1740. ^{1}H-NMR ($CDCl_3$/$(CD_3)_2CO$): δ = 0.03 and 0.13 (each 3H, s, 2 CH_3Si); 0.77 (9H, s, t-butyl); 2.7 (4H, s, $2CH_2$); 3.1 (1H, dd, J = 3.0, 15.0 Hz, $COCH_2$ trans); 3.4 (1H, dd, J = 5.7, 15.0 Hz, $COCH_2$ cis) 4.2 (1H, dd, J = 3.0, 5.7 Hz, NCH). MS: m/z: 269 (M^+-t-butyl), 228 (M^+-succinimide), 172 (M^+-t-butyl and succinimide). Anal. calc. for $C_{14}H_{22}N_2O_5Si$: C = 51.5, H = 6.7, N = 8.6, Si = 8.6 %, found: C = 51.3, H = 6.8, N = 8.9, Si = 8.5 %.

Preparation of L-valine benzyl ester-p-toluenesulfonate salt (20)

Into a round-bottomed flask were placed L-valine (5.85 g, 50 mmol), p-toluenesulfonic acid monohydrate (9.7 g, 51 mmol) benzyl alcohol (20 ml), and benzene (10 ml). The mixture was heated under reflux, with the liberated water being removed azeotropically and trapped with the aid of a Dean-Stark apparatus. A clear solution was obtained soon after reflux began. When water (about 2 ml) was distilled off (4 h) the reaction mixture was permitted to cool to room temperature, and a mixture

of benzene (50 ml) and dry ether (80 ml) was added. After standing for 2 h at 4 °C, the crystalline benzyl valinate-p-toluenesulfonate was filtered off, washed with anhydrous ether, and recrystallized from methanol-ether, gave the product (27 g, 83 %) as a white solid compound; m.p: 154-156 °C. I.R (cm^{-1}): 3250-2500, 1745, 1600, 1380, 1170, 820. ^{1}H-NMR ($CDCl_3$): δ = 0.85 and 0.89 (each 3H, d, J = 6.0 Hz, 2 CH_3); 2.17 (1H, m, C<u>H</u>$(CH)_3)_2$); 2.32 (3H, s, PhC<u>H</u>$_3$): 3.93 (1H, m, CHN); 5.0 and 5.15 (each 1H, d, J = 10.8 Hz, C<u>H</u>$_2$Ph); 7.12 and 7.81 (each 2H, d, J = 8.0 Hz, C_6H_4): 7.3 (5H, s, C_6H_5); 8.24 (3H, m, NH_3). MS: m/z: 208 (M^+-$C_7H_8SO_3$), 172 ($C_7H_8SO_3$), 116 (M^+-$C_7H_8SO_3$ and $PhCH_2$), 91 (PhC^+H_2). Anal. calc. for $C_{19}H_{25}NO_5S$: C = 60.2, H = 6.6, N = 3.7, S = 8.4 %, found: C = 60.1, H = 6.5, N = 3.7, S = 8.4 %.

<u>Preparation of N`-(3-methyl-1-benzoxybutyr-2-yl)-azetidin-2-one-4-carboxamide (22)</u>

To a solution of 1-(t-butyldimethylsilyl)-4-(succinamido-N-oxycarbonyl) azetidin-2-one (19) (2.4 g, 7.2 mmol) in 1,2-dimethyoxyethane (12 ml) were added L-valine benzyl ester-p-toluenesulfonate (2.84 g, 7.2 mmol) and N-methylmorpholine (0.8 ml, 0.736 g, 7.2 mmol). The mixture was stirred for 24 h at room temperature after which it was poured into cold water (40 ml) and extracted with ethyl acetate (2x 40 ml). The ethyl acetate fractions were bulked, dried ($MgSO_4$) and evaporated <u>in vacuo</u>. The residue was dissolved in ethanol (20 ml), concentrated hydrochloric acid (0.5 ml) was added and the mixture was stirred for 0.5 h at room temperature. The solution was diluted with ethyl acetate (100 ml) and washed with saturated sodium bicarbonate solution (30 ml) followed by saturated brine (2 x 30 ml). The organic fraction was dried (Na_2SO_4) and evaporated. The residue was chromatographed (gradient elution; 1:1 ethyl acetate/ toluene to ethyl acetate) to provide the product as a colorless oil which crystallized from petroleum ether (b.p. 40-60 °C) as a white solid (1.45 g, 65 %); m.p: 78-79°C. I.R (cm^{-1}): 3280, 1758, 1735, 1664. ^{1}H-N.M.R. ($CDCl_3$): δ = 0.87 and 0.93 (each 3H, d, J= 6.7 Hz, 2 CH_3); 2.24 (1H, m, C<u>H</u>$(CH_3)_2$); 3.02 (1H, dd, J = 2.7, 15.2 Hz, $COCH_2$ <u>trans</u>); 3.36 (1H, m, $COCH_2$ <u>cis</u>), 4.19 (1H, m, C<u>H</u>CH$(CH_3)_2$); 4.64 (1H, m, CH_2C<u>H</u>); 5.14 and 5. 24 (each 1H, d, J= 12.2 Hz, C<u>H</u>$_2$Ph); 6.7 and 6.87 (each 1H, br., 2NH); 7.39 (5H, s, Ph). MS: m/z: 304 (M^+); 213 (M^+-CH_2Ph), 169 (M^+-CO_2CH_2Ph), 127[M^+-$CH(CH_3)_2$ and CO_2CH_2Ph], 115 [M^+-$(CH_2)_2CHC^+HCO_2CH_2Ph$), 91 ($PhCH^+_2$). Anal. calc. for $C_{16}H_{20}N_2O_4$: C = 63.2, H = 6.6, N = 9.2 %, found: C = 63.4, H = 6.9, N=9.4 %.

<u>Preparation of N'-(3-methyl-1-hydroxybutyr-2-yl)-azetidin- 2-one-4-carboxamide (11)</u>

N'-(3-methyl-1-benzoxybutyr-2-yl)-azetidin-2-one-4-carboxamide (22) (1.0 g, 3.2 mmol) was dissolved in ethanol (50 ml) and palladium on activated charcoal (0.064 g, 10%) was added. The mixture was hydrogenated for 3 h at one atmosphere after which it was filtered and the solvent was evaporated. Chromatography of the residue (acetone/ethyl acetate 1:1) provided the product (0.68 g, 97%) as a white crystalline solid; m.p: 201-203°C. I.R (cm^{-1}): 3650-2300, 3275, 1740, 1650, 1540. ^{1}H-NMR (DMSO-d_6): δ = 0.95 and 1.15 (each 3H, d, J = 5.0, 2.18 Hz [1H, m, $CH(CH_3)_2$]; 2.76 (1H, d (br.), J= 17.0 Hz, $COCH_2$ <u>trans</u>); 3.2 (1H, dd, J = 4.7, 17.0 Hz, $COCH_2$ <u>cis</u>); 4.26 (2H, m, 2 NCH); 8.3 (2H, m, 2 NH). MS: m/z: 214 (M^+), 171 [M^+-$CH(CH_3)_2$], 169 (M^+ - CO_2H), 114 [M^+-$(CH_3)_2CHC^+HCO_2H$]. High Resolution MS for $C_9H_{14}N_2O_4$: M^+ = 214.0954, found M^+ = 214.0965. Anal. calc. for $C_9H_{14}N_2O_4$: C= 50.4, H= 6.5, N= 13.1%, found: C= 50.2, H= 6.8, N = 13.1 %.

3. RESULT AND DISCUSSION

In this particular investigation, we wish to record the total synthesis of compound (2) from its precursors L-valine (3) and L-aspartic acid (6) (Scheme 1), and compare its spectroscopic data, physical properties and biological activity with those reported (Shimi and Fathy, 1981).

It is clear that both precursor, L-valine and L-aspartic acid, have two reactive sites, the acid groups and the amino groups. For a required dipeptide to form the described amino acids and ensure that the peptide links occur in the required places, the amino group of L-valine has to be protected and the two acid groups of L-aspartic acid have to be protected.

In the first stage, the amino group of L-valine (3) was protected and the carbobenzoxy group was chosen for this. Protection was achieved by the concurrent addition of carbobenzoxy chloride and sodium hydroxide solution to a solution of L-valine in aqueous sodium hydroxide at 0 °C. After work-up the required product (4) was obtained (Schwarz *et al*, 1957).

On the other hand, the acid group of the valine was activated by reaction with N-hydroxy-succinimide in dioxane and in the presence of DCC to give compound (5).

The two carboxy groups of L-aspartic acid were both protected as their methyl esters (Greenstein and Winitz, 1961). This was effected by careful addition of thionyl chloride to methanol, after which L-aspartic acid (6) was added. After stirring for 60 hours at room temperature, the required aspartate (7) was obtained in 63 % yield.

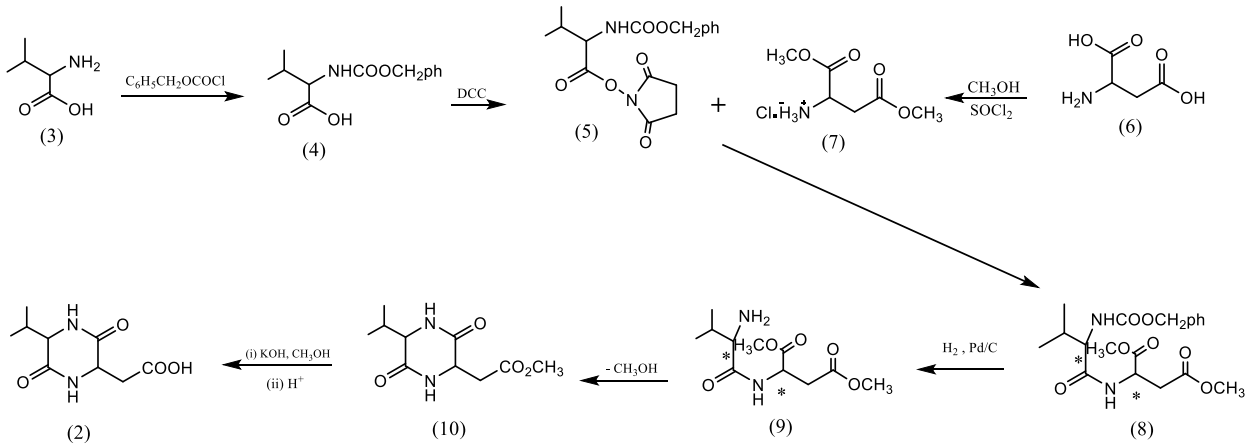
The required dipeptide (8) was synthesized by reacting compound (5) with compound (7) in the presence of N-methylmorpholine, which was used as base in order to reduce the chance of racemization occurring at the chiral carbon *****C (Cavelier *et al*, 2001).

The first problem to be overcome was the development of an efficient approach for cyclization of dipeptide (8), in which the benzyl moiety was cleaved from its amide linkage. It was decided to use the acetic acid-catalyzed diketopiperazine synthesis (Suzuki *et al*, 1981). The approach involved cleavage with hydrogen bromide in acetic acid. The mixture was refluxed with stirring and the crystals were obtained by evaporation. The yield was poor and the n.m.r. spectrum showed that the carbobenzoxy group had not been totally cleaved.

Our attention then turned to another approach which was palladium catalysed hydrogenolysis. A solution of dipeptide (8) in a mixture of butane-2-ol and acetic acid was hydrogenated in the presence of palladium on activated charcoal as a catalyst. The desired cyclic compound (10) was obtained in 92 % yield.

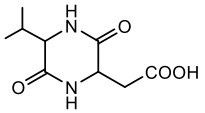
The final stage involved the removal of the protecting methyl group to give the target compound (2). The cyclized compound (10) was dissolved in methanol and an aqueous solution of potassium hydroxide was added. On work-up, the desired piperazine (2) was obtained in 75 % yield (Scheme 1).

All spectroscopic data, and elemental analysis confirm that the synthesized compound was 6-isopropyl-2,5-diketopiperazine-3-acetic acid. The antibacterial testing results of the compound (2), which were carried out at Glaxo are shown in Table 1. It exhibited no activity against the 27 bacterial and fungal strains that formed the test.



Scheme 1

Table 1. Glaxo Group Research LTD: Microbiology Division (AM-6 Antimicrobial Screen)

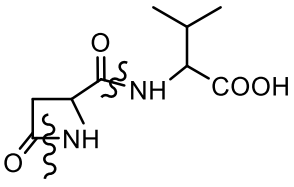
Amount: 100 mg		Compound No: GB 35538X		Solubility: $NaHCO_3$				
Antibacterial Activity 10^3 or 10^5 efu				Structure: Cairomycin A 				
Organism	**No.**	**Test**	**CER**					
Staph. aureus 853E	1	>500	≤ 0.06					
Staph. aureus C864	2	>500	≤ 0.06					
Staph. epidermidis 887E	3	>500	≤ 0.06					
Micrococcus sp. 1810 E	4	>500	≤ 0.06					
Strept. faecalis 850	5	>500	4					
B. subtilis 841	6	>500	≤ 0.06					
B. cereus 173 BE	7	>500	0.5					
E. coli 851E	8	>500	2					
E. coli DC0 1850E	9	>500	2	Antifungal activity MIC mcg/ml				
E. coli DC0 1852E	10	>500	1					
E. coli C2119	11	>500	2	**Organism:**	**No.**	**Test**	**Amph B**	**Acute Mouse Tox**
Ent. cloasae 1321E	12	>500	4					
K. aerogenes 1522E	13	>500	1	M. canis	764E	>500	1	
K. pneunoriae 466	14	>500	2	T. mentag	687E	>500	1	
Serr. maraescens 1324E	15	>500	62	T. verrucosum	2126E	>500	62	
Ps. aerucinosa 1371E	16	>500	> 125	T. rubrum	1812E	>500	0.5	
Ps. aerucinosa 165 SAI'	17	>500	125	A. niger	C116	>500	2	
C. albicens C316	18	>500	> 125					
C. albicens 120BE	19	>500	> 125	In vivo evaluations				
Pr. Nirabilis 431E	20	>500	4	Antibacterial:				
Pr. morganii NCTC 235	21	>500	31	Antifungal:				
Providence sp. 1497E	22	>500	125	Antiprotozoal:				
Bact. fragilis* 9326	24	>500	125					
Bact. fragilis* 1602E	25	>500	> 125					
CL. perfringens* 2045E	26	>500	1					
Cl. sporogenes* 2052E	27	>500	1					

MIC`s >125µg/ml vs 25 Yeasts (experiment records IV book 278 p 58)

When the data of the synthesized piperazine was compared with that of published cairomycin A, it showed that they are two different compounds.

Cairomycin A had a melting point 110-112 ºC, while the synthesized piperazine was melted at 222-224 ºC. Infrared absorption of cairomycin A has a strong band at 1030, 1625 and 1735 cm^{-1}, whereas that of the synthesized compound showed strong absorption band at 1725 cm^{-1} and broad absorption band at 1645 cm^{-1}. A clear difference was observed in their n.m.r. spectra (Table 2); cairomycin A was run in $CDCl_3$ while the synthesized piperazine was not soluble in $CDCl_3$ or $(CD_3)_2CO$, and was run in DMSO-d6. In cairomycin A, a signal at δ 5.51 (triplet) was assigned to the methine proton of (CH-CH_2), whereas in our compounds this was at δ 4.11. Another difference which was most important was that while cairomycin A possessed potent activity against Gram-positive bacteria, the synthesized piperazine was biologically inactive.

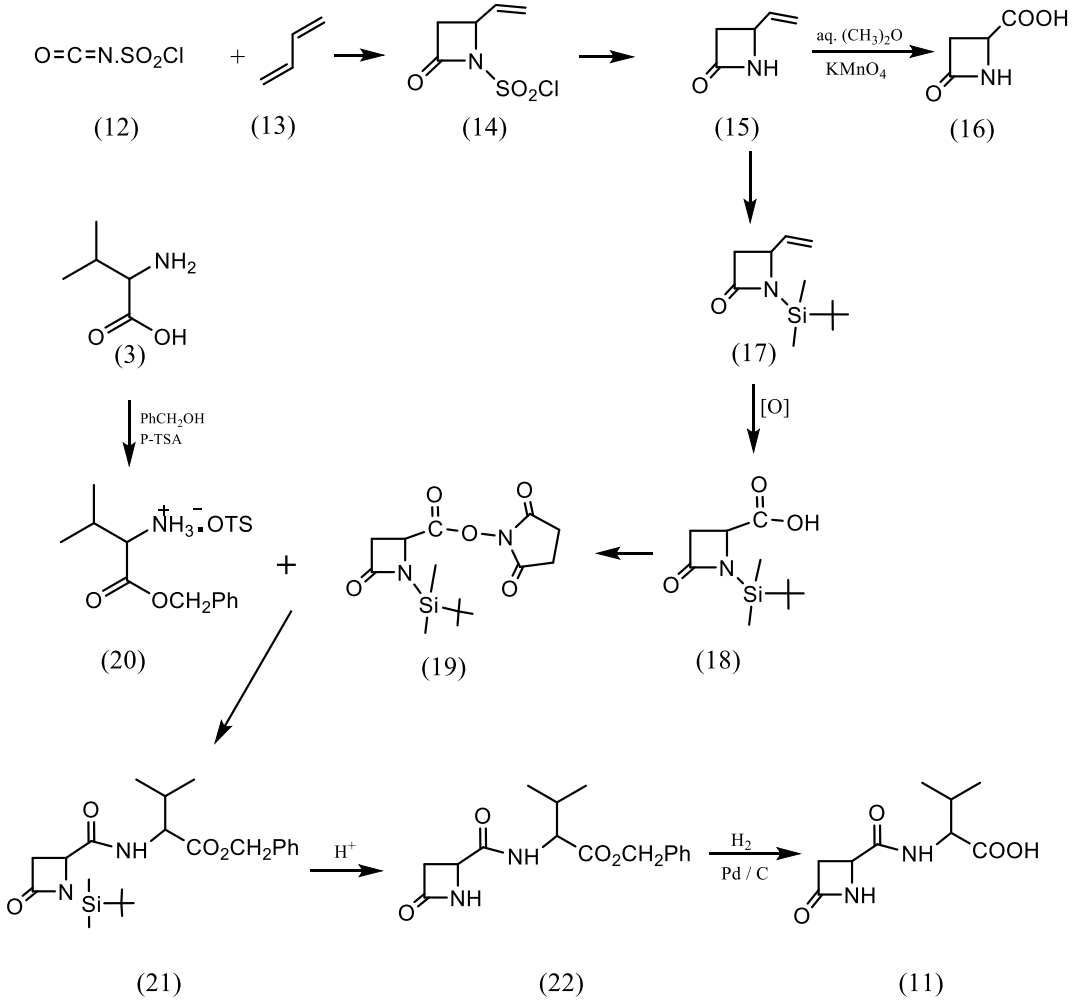
From the above evidence, it can be concluded that the structure of cairomycin A is not 6-isopropyl-2,5-diketopiperazine-3-acetic acid as proposed. Since acid hydrolysis of cairomycin A produced L-valine and L-aspartic acid, then they must be condensed together in another way to that proposed. We concluded that another possibility for its structure was one with β-lactam skeleton (11) and composed from two required amino acids valine and aspartic acid.



L-aspartic acid L-valine

(11)

Thus, the β-lactam compound (11) became our target and which was hoped to be cairomycin A. The starting material chosen for this work was 4-vinylazetidin-2-one (15). This compound has already been synthesized by Stoodley (Brennan et al, 1980). The azetidinone (15) was obtained by the cycloaddition of chlorosulphonyl isocyanate (12) with 1,4-butadiene (13) to give N-sulphonyl chloride (14) which was reduced, without isolation, to 4-vinylazetidin-2-one (15) by treating with sodium sulphite. This method was improved, by addition of sodium carbonate to the reaction mixture to destroy the hydrogen chloride which was present in the chloro-sulphonyl isocyanate (Scheme 2).



Scheme 2

Moriconi has suggested (Moriconi and Meyer, 1971) a mechanism for the cycloaddition reaction, in which he proposed that the reaction starts with π complex formation and proceeds through the polar transition state.

The first problem to be overcome was the development of an efficient method for oxidation of the vinyl group. This oxidation has already been done (Pietsch, 1976); oxidation of vinylazetidin-one (15) with potassium permanganate in aqueous acetone provided 4-hydroxycarbonylazetidin-2-one (16) in 40 % yield.

In order to develop an improved procedure to increase the yield of azetidinone (16), a variety of oxidizing agents was tried in a variety of solvents. The oxidizing agents used were: ozone (Vollhardt and Schore, 2007), potassium permanganate (Wang *et al*, 2015), pyridinium dichromate (Corey and Schmidt, 1979), and periodate plus permanganate (Lemienx and Rudloff, 1955); in a variety of solvents; methanol, dichloromethane, DMF, acetone or water. These attempts all were unsuccessful, and generally the yield was very poor. It was concluded that the problems arose in isolating the product because its solubility in the aqueous phase, while its solubility in the organic solvents was quite low. In an attempt to overcome the problem, it was decided to protect the β-lactam nitrogen. The protecting group chosen was tertbutyldimethylsilyl in the hope of producing an organic-soluble product.

N-silylation of the vinylazetidinone (15) with tert-butyldimethylchlorosilane led to azetidinone (17) in 89 % yield. The azetidinone (17) was oxidized with potassium permanganate in acetone, at room temperature, to give the required N-protected azetidinone (18) in 85 % as a white solid. In the next step the acid function in the compound (18) was activated by the N-hydroxy-succinamide group. The required

azetidinone (19) was synthesized by reacting azetidinone (18) with N-hydroxysuccinamide in the presence of DCC, using dioxane as a solvent.

On the other hand, the acid site of the valine was protected with a benzyl group, while its amine site was temporarily protected as its *p*-toluene sulphonate salt in order to ultimately react the required amine site of valine with acid site of azetidinone (19) and to obtain the required peptide linkage.

The desired valine ester (20) was synthesized by reacting valine with benzyl alcohol in the presence of *p*-toluenesulphonic acid (Zervas *et al*, 1957). The resulting compound (20) was obtained in 83 % as a white solid.

As this stage, both moieties of our target compound (21), azetidinone (19) and valine (20), are ready and the next step will be their reaction together in order to obtain the desired azetidinone (21). The required azetidinone (21) was thus obtained by reaction of the azetidinone (19) with valine ester (20) in 1,2-dimethoxyethane and the presence of N-methyl morthine.

It was noted, during purification of compound (21) by chromatography, that the partial desilylation took place and a mixture of N-protected and N-deprotected azetidinone was obtained. Therefore, the crude azetidinone (21) was treated with concentrated hydrochloric acid in ethanol to give the totally N-deprotected azetidinone (22) in 65 % yield (from the azetidinone (19)) as a white solid.

The azetidinone (22) was next hydrogenated in the presence of palladium on activated charcoal as a catalyst to provide the desired target (11) in 97% yield.

The biological activity of compounds (11) against 27 bacterial and fungal strains were carried out at Glaxo. The testing results indicated that the synthesized β-lactam compound (11) was neither antibacterial nor β-lactamase inhibitory. The physical properties and spectroscopic data of the synthesized azetidinone (1) were compared to those published for cairomycin A (Shimi and Fathy, 1981).

Cairomycin A is described as a yellowish brown powder which melted at 110-112 ºC, while the synthesized azetidinone was obtained as a white solid with melting point of 201-203 ºC. Cairomycin A was freely soluble in chloroform, ethyl acetate, acetone and benzene; whereas the azetidinone (11) was insoluble in all but soluble in DMSO. The carbonyl group absorption of cairomycin A was at 1735 cm^{-1}, while the analogues β-lactam carbonyl absorbed at 1740 cm^{-1}.

A clear difference also observed when their n.m.r. spectra (Table 2) were compared, was the peak at δ 5.51 (triplet) which was proposed for methine proton of (C<u>H</u>-CH_2), whereas in the β-lactam isomer this was δ 4.26. This difference in n.m.r. spectra was strong evidence to say that the suggested β-lactam structure for cairomycin A is doubtful. Another important difference between the isolated cairomycin A and the synthesized β-lactam compound (11) was the difference in their biological activity. As described before, cairomycin A possessed potent activity against Gram-positive bacteria, while the synthesized β-lactam compound was biologically inactive. From the evidence above, it would be concluded that the synthesized azetidinone (11) is not the structure of cairomycin A.

Table 2. ^{1}H-NMR Spectra Comparison

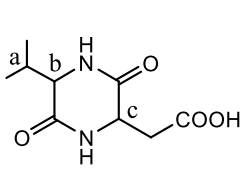
Cairomycin A			Compound (2)			Compound (11)		
δ	Mult.	Assig.	δ	Mult.	Assig.	δ	Mult.	Assig.
1.02	d	$2CH_3$	0.83	d	CH_3	0.95	d	CH_3
1.52	h	Ha	0.91	d	CH_3	1.15	d	CH_3
3.04	d	CH_2	2.1	m	Ha	2.18	m	Ha'
4.25	d	Hb	2.65	d		2.76	dd	
5.51	t	Hc	2.7	d	CH_2	3.20	dd	CH_2
9.05	br.	2NH	3.58	m		4.26	m	
9.2	br.	OH	4.11	m	Hb	8.3	m	Hb' & Hc'
			8.04	s	Hc			2NH
			8.12	s	NH			
					NH			

Table 3. Mass Spectra Comparison

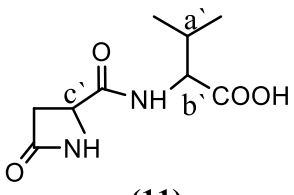
Cairomycin A		Compound (2)		Compound (11)	
m/z	%	m/z	%	m/z	%
214 (M^+)	15	214 (M^+)	50	214 (M^+)	20
144	87	172	66	172	34
111	100	126	58	151	54
99	40	112	100	135	63
84	73	88	40	126	27
70	63	72	65	100	11
57	30	60	18	88	100
55	25	55	62	74	25
43	79	43	45	45	7
41	24	29	58	28	31

4. CONCLUSION

The synthesis of proposed structure of cairomycin A, 6-isopropyl-2,5-diketopiperazine-3-acetic acid (2), and its β-lactam isomer (11) where studied.



(2)



(11)

When the physical properties, spectroscopic data and biological activities of both synthesized compounds (2) and (11) where compared with those for cairomycin A, it was concluded that the structure of cairomycin A neither 6-isopropyl-2,5-diketopiperazine-3-acetic acid (2) nor its β-lactam analogues (11).

The n.m.r. cairomycin A was run in $CDCl_3$, while the synthesized compounds were not soluble in $CDCl_3$ and were run in DMSO-d6.

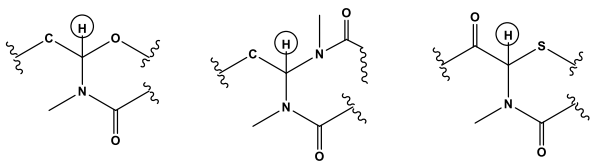
It has been reported that the Hc in the cairomycin A had a signal at δ 5.51 which would be too high for such a type of proton.

An N.O.E. difference experiment in which H_C was irradiated showed equal effects on both H_d Protons indicating that these had free rotation relative to H_C.

According to the above information it was concluded that the methine proton (Hc) could be neighboring an electron-rich atom such as oxygen or sulfur. The displacement of two oxygen atoms (in $C_9H_{14}N_2O_4$) by one sulfur atom gives a structure with the same molecular weight 214 (Table 3).

After it was proved that the structure of cairomycin A was neither (2) nor (11), it was concluded that the methine group (CHc) in the cairomycin A, might be surrounded by one of the following proposed groups, to have such chemical shift (δ 5.51):

i) An amide group and an oxygen atom;
ii) Two amide groups;
iii) An amide and carbonyl group, and a sulfur atom.



(i) (ii) (iii)

Further investigation of this problem would however, require a pure sample of the substance designated cairomycin A.

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