Synthesis and high field NMR study of a new cyclodipeptide–β-cyclodextrin derivative

Giuseppe Impellizzeri,[a] Giuseppe Pappalardo,[b] Enrico Rizzarelli[a,b] and Corrado Tringali[a]

[a] *Dipartimento di Scienze Chimiche, Università di Catania, Viale A. Doria 6, 95125 Catania, Italy*

[b] *Istituto per lo Studio delle Sostanze Naturali di Interesse Alimentare e Chimico-Farmaceutico, CNR, Viale A. Doria 6, 95125 Catania, Italy*

The synthesis and high field NMR study of a new cyclopeptide functionalized-β-cyclodextrin β-CDen-c-(Glu-Glu) (3) in aqueous solution are reported. This compound has been synthesized by condensation of the ethylendiamine-β-cyclodextrin derivative β-CDen (1) with the cyclo-(glutamyl-glutamyl) [c-(Glu-Glu)] (2). The NMR analysis has been carried out on 500, 600 and 750 MHz instruments and has been largely based on advanced two-dimensional NMR experiments, *i.e.* DQF-COSY, TOCSY, HMQC and HMBC. The selective excitation technique (1D TOCSY) has also been applied. The study has led to a complete ^{1}H and ^{13}C NMR assignment of the pendant moiety and the modified glucopyranose unit (A), and a detailed assignment of the unmodified glucopyranose units (B–G). Data about the preferred conformation of 3 are also acquired by means of ROESY experiments.

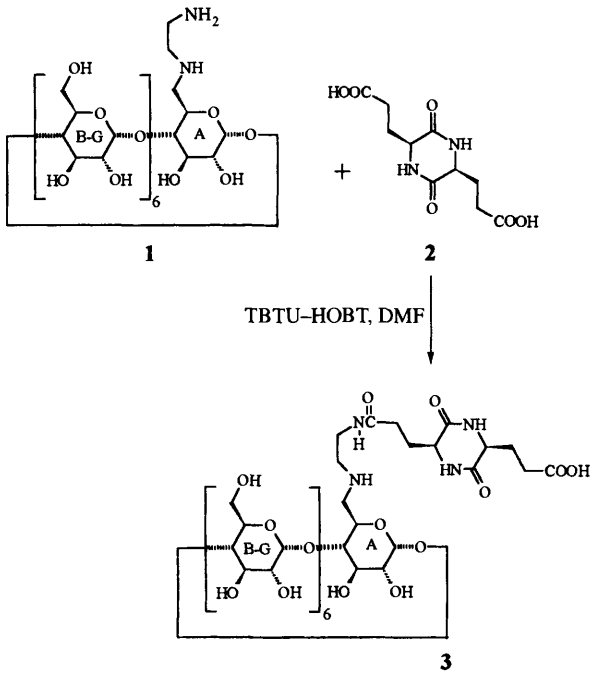
Introduction

Cyclodextrins (CDs) are cyclic oligosaccharides consisting of six or more α-(1→4)-linked glucopyranose units, which are produced by the action of *Bacillus macerans* amylase on starch. CDs containing six, seven and eight glucopyranose units, are specified as α-, β- and γ-CD respectively.[1,2] The hydrophobic interior of these torus-shaped molecules allows the complexation of organic molecules of appropriate size in aqueous solution.[3] This ability has made it possible to utilize CDs for many applications,[4] including the complexation and transport of hydrophobic drugs for pharmaceutical purposes.[5] However, CDs lack any other biological recognition sites apart from their cavity and the transport of the included drug is not specific. The chemical modification of CDs with appropriate functional groups capable of interacting with specific biological receptors, may lead to the production of new vectors, for the delivery *in vivo* of pharmacologically active compounds. A variety of bioactive molecules have been grafted, with this purpose, onto CDs;[6] among them, peptides are an important example, mainly due to the role that amino acid side-chains play in receptor recognition.[7] However, only a few reports have appeared on the derivatization of CDs with cyclopeptides,[8] despite the fact that cyclic peptides display an array of biological activities as hormones, toxins, antibiotics and regulators of ion transport.[9,10] In particular, cyclic peptides containing complexing side-chain substituents, such as imidazole or carboxylate, have been extensively studied in view of their capacity to mimic the active site of metallo enzymes in coordinating metal ions.[11] Cyclopeptides bearing carboxy groups have been synthesized as models for ionophores which transport metal ions through biological membranes.[12]

L-Glu is one of the major excitatory neurotransmitters in the mammalian brain[13] and some Glu-based oligopeptides, particularly peptides with a γ-glutamyl linkage, have been isolated from mammalian brain, spinal cord and other nervous tissues.[14] In addition, it has been recently reported that spaglumic acid (*N*-acetyl-L-aspartyl-L-glutamic acid), a dipeptide occurring in mammalian brains, is easily cyclized in aqueous solution to give the corresponding diketopiperazinedicarboxylic acid.[15] This fact has been emphasized in view of the possibility that spaglumic acid, as well as other peptides, could cyclize in the body fluids and exert biological activity in this form.[15] Finally, in the field of neuropharmacology, considerable interest in L-Glu rigid analogues has been observed.[16] Thus, we decided to graft the diketopiperazine of L-glutamic acid onto a β-CD. In order to obtain a more flexible model, useful for the study of the mutual interaction that could arise when the two parts are linked together, we have inserted an ethylendiamine 'spacer' between the β-CD torus and the cyclopeptide moiety.

In the present paper, we report the synthesis and, in as much detail as possible, the high field NMR study of the new cyclopeptide functionalized β-CD: 6-deoxy-6-(2-{3-[(2S,5S)-5-carboxyethyl-3,6-dioxopiperazin-2-yl]propionamido}ethylamino)cyclomaltoheptaose, designated β-CDen-c-(Glu-Glu) (**3**).

Unsymmetrically modified β-CDs usually give very complicated ^{1}H NMR spectra, and even proton-decoupled ^{13}C NMR spectra show severe overlapping; this is essentially due to the magnetic inequivalence of the seven glucopyranose units, which give rise, in principle, to 49 distinct ^{1}H NMR signals, disregarding the exchangeable protons. In addition, the signals related to the introduced functional groups are obviously present. Proton-decoupled ^{13}C NMR spectra, are less complicated, including 42 virtually inequivalent singlets for the β-CD moiety: thus, the use of heteronuclear correlated two-dimensional NMR experiments can be a valuable aid in the assignment of the proton resonances. Nevertheless, the majority of the NMR studies on modified CDs, have been carried out on the basis of ^{1}H NMR spectroscopy and, to the best of our knowledge, a complete assignment of both ^{1}H and ^{13}C NMR signals has never been accomplished. Of course, the situation is even more complicated if some of the signals due to the pendant overlap with the CD resonances, as in the present case. Thus, for the NMR study of β-CDen-c-(Glu-Glu), we resorted to an integrated use of the higher fields available (500, 600 and 750 MHz) and the more advanced one- and two-dimensional NMR techniques: as shown below, this led to a complete ^{1}H and ^{13}C NMR assignment of the signals associated with both the pendant moiety and the modified glucopyranose unit (A), along with an assignment, that is as detailed as possible, of the unmodified glucopyranose units (B–G).



Scheme 1

Results and discussion

Synthesis

The title compound β-CDen-c-(Glu-Glu) (**3**) was synthesized by condensation of 6-deoxy-6-(2-aminoethylamino)-β-cyclodextrin (β-CDen, **1**)[17] with (3*S*,6*S*)-3,6-bis(3-carboxyethyl)-2,5-dioxopiperazine [c-(Glu-Glu), **2**] in DMF under standard peptide synthesis conditions[18] (Scheme 1). Purification by ion exchange chromatography afforded the desired product in 60% yield. The FAB mass spectrum gave the expected molecular ion peak. A crucial point in establishing structure **3**, was unambiguously confirmation that the cyclopeptide moiety was bound, as projected, to the terminal amino group of the ethylenediamino spacer. The alternative structure, deriving from the reaction of the amino group in 6A with the c-(Glu-Glu), should have a fully substituted amide and a free NH_2 group. The assignment was achieved through a preliminary ^1H NMR analysis; in particular, we carried out some experiments in [$^2H_6$]dimethyl sulfoxide ([$^2H_6$]DMSO) solution in order to observe the NH protons. The D_2O-exchangeable signals at δ 8.21 (1 H) and 8.15 (1 H), in this spectrum, were assigned, on the basis of their chemical shift value and COSY experiments, to the two amide protons of the dioxopiperazine ring (see Experimental section); a third D_2O-exchangeable downfield signal was observed as a broad 1 H triplet at δ 7.84. The further NH signal, expected for structure **3**, was not discernible in the spectrum; nevertheless, by addition of dry CF_3COOH, a broad D_2O-exchangeable, 2 H signal centred at δ 8.52, was observed, while the above cited 1 H peaks, showed only a slight downfield shift. This clear evidence in favour of structure **3**, was corroborated by the following observations: three methylene signals resonating at δ 2.59, 2.83 and 3.12, before addition of acid could be assigned, on the basis of the spectral analysis aided by the COSY experiment, to ζ-CH_2, 6A-CH_2 and ε-CH_2, respectively. In the ^1H NMR spectrum of the protonated sample, a neat downfield shift was observed for the signals due to ζ-CH_2 and 6A-CH_2; in contrast, the signal due to ε-CH_2 remained almost unaffected.†

† The numbering system used in the assignment of NMR signals for **3** is shown in Fig. 1.

Table 1 ^1H and ^{13}C NMR data for β-CDen-c-(Glu-Glu) (**3**)[a]

Position	δ_H	δ_C
1A	5.19	103.8
2A	3.74	74.9
3A	4.06	75.4
4A	3.59	85.7
5A	4.19	70.1
6A	3.39	50.9
6′A	3.62	50.9
ζ	3.27	50.9
ε	3.60	38.5
γ′		178.7
β′	2.43, 2.47	33.2
α′	2.24	31.3
6′	4.29	56.5
5′		172.0
3′	4.27	56.8
2′		172.5
α	2.18	32.6
β	2.35	35.2
γ		183.6
1B-G	5.15	104.4
2B-G	3.73	74.4[b]
3B-G	3.99–4.04	75.4, 75.7
4B-G	3.61–3.69	83.1, 83.7, 84.0
5B-G	3.92	74.7[b]
6B-G	3.84–4.02	62.9, 63.3

[a] Run at 750.13 (^1H) and 125.69 (^{13}C) MHz, in D_2O. Chemical shifts are in ppm and refer to the residual water peak assigned at 4.8 ppm. Fig. 1 shows the numbering system used for the assignments. [b] Interchangeable values.

High field NMR study

In order to simplify the ^1H NMR spectrum of **3**, the majority of the experiments have been carried out in D_2O, on a 5.3 mmol dm^{-3} sample, where all the exchangeable protons were substituted by ^2H nuclei. To achieve the best possible resolution, a 750 MHz instrument has been used for the one-dimensional ^1H NMR spectrum of **3** (Fig. 1). Thus, the chemical shifts listed in Table 1, have been determined from this spectrum. Even at 750 MHz, the ^1H NMR spectrum of **3**, is very complicated: the signals due to 63 protons appear, with partial overlapping, within a *ca.* 3 ppm region. The proton-decoupled ^{13}C NMR spectrum (Fig. 2), has been taken at 125.69 MHz, and clearly shows overlapped peaks in the glucopyranose region. The assignments of ^1H and ^{13}C resonances, have been aided by a combined use of one- and two-dimensional NMR techniques (1D DEPT,[19] 1D TOCSY,[20] DQF-COSY,[21] TOCSY,[22] HMQC,[23] HMBC[24]), carried out on 500 and 600 MHz instruments; indeed, a careful analysis of the 500 MHz ^1H NMR spectrum (top trace in the HMQC spectrum, Fig. 3), showed that the loss in apparent resolution from the 750 to the 500 MHz spectrum, was negligible in comparison with the inherently low resolution of the two-dimensional spectra.

The first step in the spectral analysis, was the assignment of all the ^1H and ^{13}C resonances due to the propionamido-c-(Glu-Glu) moiety. A convenient starting point was the ^{13}C carboxy peak at δ 183.6, easily assignable on the basis of its high chemical shift value. This showed long-range correlation (HMBC) with a methylene signal (δ 2.35), that was HMQC-correlated with the β-carbon at δ 35.2; this was in turn long-range heterocorrelated with the signals at δ 2.18 and 4.27, the latter assigned to the 3′ methine of the dioxopiperazine ring. The HMQC spectrum allowed identification of the related carbons respectively at δ 32.6 (C-α) and 56.8 (C-3′); further spectral data from the DQF-COSY, TOCSY (Fig. 4) and HMBC spectra, were in agreement with these assignments; the carbonyl peak at δ 172.5 (C-2′) was assigned on the basis of the HMBC cross-peak with H-3′. Further assignments of the dioxopiperazine resonances were straightforward: the methine at δ 56.5 (C-6′), one-bond correlated with the signal at δ 4.29 (H-6′),

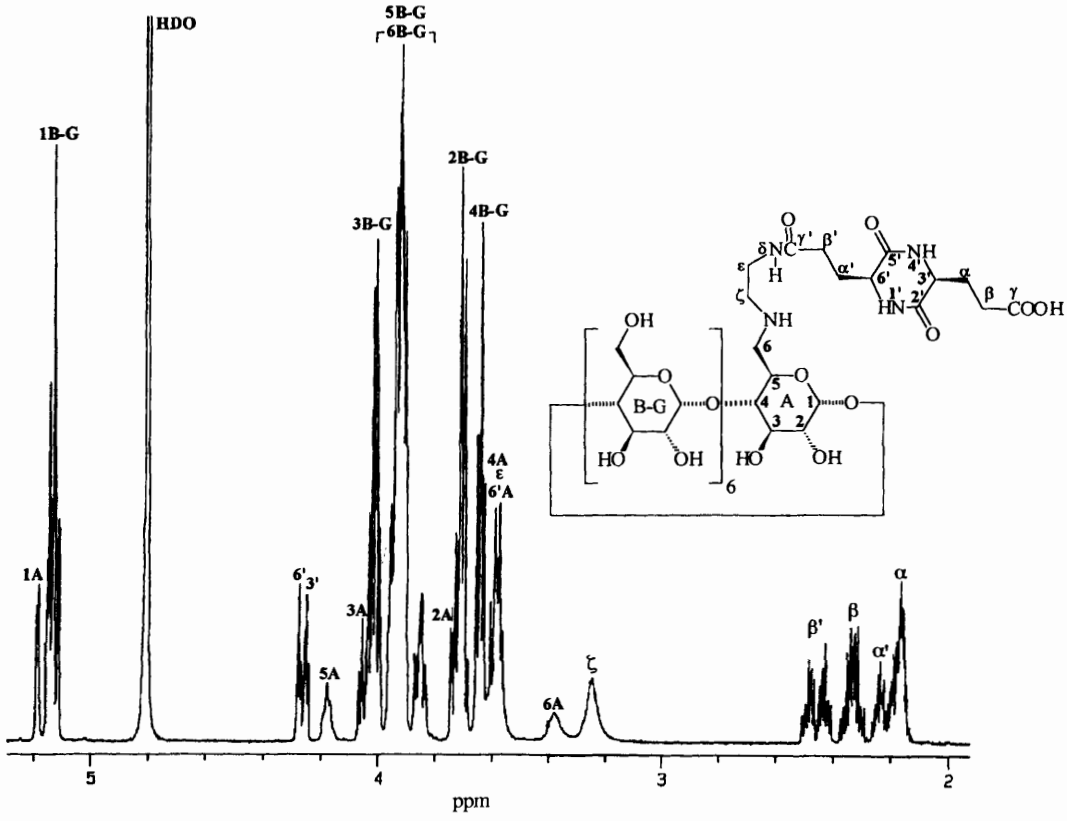


Fig. 1 750 MHz ^{1}H NMR spectrum of **3** in D_2O

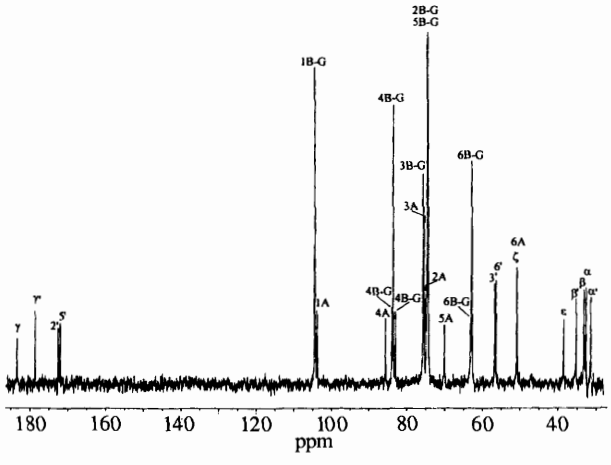


Fig. 2 125.69 MHz proton-decoupled ^{13}C NMR spectrum of **3** in D_2O

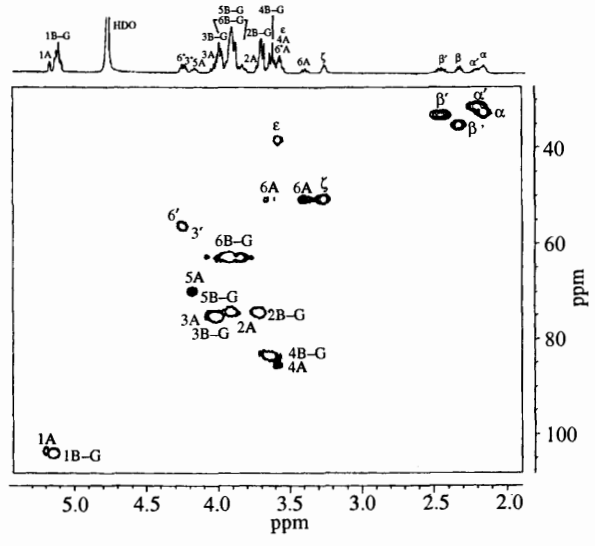


Fig. 3 500 MHz ^{1}H–^{13}C HMQC spectrum of **3** in D_2O. Top trace: 500 MHz ^{1}H NMR spectrum of **3** in D_2O.

and the carbonyl at δ 172.0 (C-5′), long-range correlated with H-6′. Further data from two-dimensional NMR spectra allowed assignment of the carbon and proton resonances at positions α′, as reported in Table 1; the β′-methylene resonances appeared as distinct multiplets at δ 2.43 and 2.47, one-bond correlated with the carbon signal at δ 33.2; the β′ proton signals, showed long-range connection to the amidic carbon at δ 178.7, in turn correlated (HMBC) with a proton signal at δ 3.60, in a crowded region including glucopyranose signals. The HMQC spectrum showed for this complex multiplet two distinct cross-peaks: one was related with a set of glucopyranose carbon resonances between δ 83.1 and 85.7, the other was due to a signal at δ 38.5, consequently assigned at ε-CH_2. The peak at δ 3.60 was coupled (DQF-COSY) only with a methylene resonance at δ 3.27, the latter, being correlated (HMQC) with the peak at δ 50.9, due to the ζ-CH_2, vicinal to the amine nitrogen.

The next step of the analysis, was the assignment of the resonances due to the glucopyranose unit A. This was accomplished starting from the anomeric proton, which appears in the ^{1}H NMR spectrum of **3** as an isolated low-field doublet (δ 5.19), and tracing the propagation of the correlation through the ring by means of the selective excitation technique (1D TOCSY). The isolated H-1, was selectively excited and the mixing time was gradually increased to follow the propagation of correlation to H-2 (δ 3.74), H-3 (δ 4.06), H-4 (δ 3.59) and H-5 (δ 4.19). Protons at position C-6 were not easily discernible; thus, H-5 was selectively excited and this further 1D TOCSY (Fig. 5) experiment allowed the identification of H-6 and H-6′ resonances (δ 3.39, 3.62) and corroborated the above cited assignments. The application of this technique, proved to be very useful in this case because it allowed the observation of the multiplet structure for overlapped protons (*i.e.* H-2, H-3, H-4) and the measurement of their coupling constants,‡ consequently

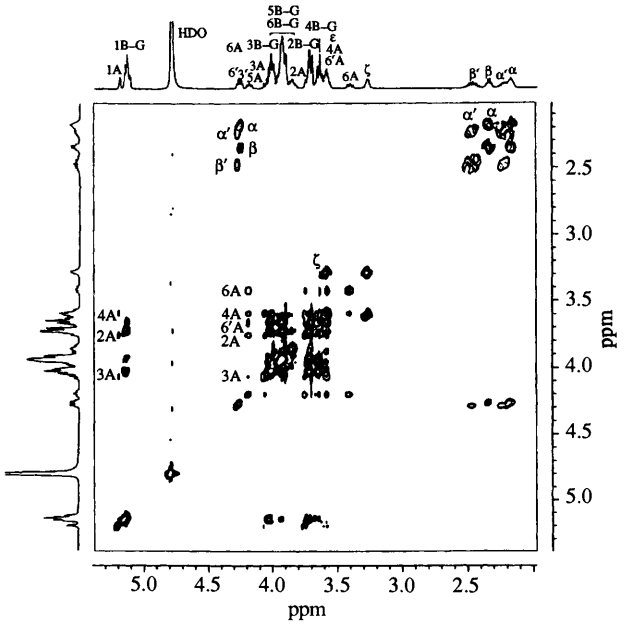


Fig. 4 500 MHz 2D TOCSY spectrum of **3** in D_2O

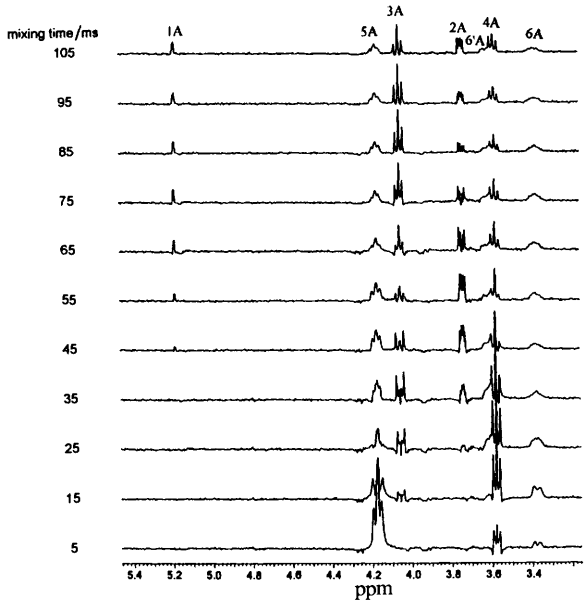


Fig. 5 500 MHz selective excitation (1D TOCSY) spectra of **3** in D_2O. The 5A proton at 4.19 ppm has been selectively excited using a Gaussian soft pulse of 80 ms and an array of mixing times from 5 to 105 ms.

giving further confirmation of the exact assignments of individual proton signals. Once the ^1H resonances of ring A had been assigned, the correlated carbon signals were detected through the HMQC spectrum, as reported in Table 1. The assignments were aided by the analysis of the intensity of the ^{13}C peaks, and the overlapping of two carbon resonances at δ 50.9 (C-ζ and C-6) was observed. Final confirmation of the assignments came from the analysis of 2D NMR spectra, *i.e.* DQF-COSY, TOCSY and HMBC.

At this point, the remaining unassigned resonances were due to the glucopyranose units B–G. Looking at this more complex part of the spectra, it was evident that the absence of aromatic nuclei in the peptide moiety causes smaller shielding effects than in other substituted cyclodextrins;[6f,25] for this reason the majority of the ^1H NMR signals, associated with this part of the molecule appear as broad complex multiplets, without any significant separation even at 750 MHz. However, some considerations can be made on the basis of a careful, integrated analysis of all the NMR spectra registered for **3**; in particular, the combined use of HMQC and DQF-COSY experiments, allowed the assignment of 'medium' chemical shift values for the glucopyranose rings B–G. The H-1 signals, are easily identified as the multiplet centred at δ 5.15; the HMQC-related carbon signal appears at δ 104.4, without an observable dispersion of the chemical shifts. The DQF-COSY spectrum shows the correlation between H-1 and a broad double doublet centred at δ 3.73 (H-2), in turn correlated with a complex entanglement of signals at δ 3.99–4.04 (H-3). The HMQC spectrum shows the related carbon resonances at δ 74.4, 74.7 (C-2, exchangeable with C-5) and δ 75.4, 75.7 (C-3), respectively. Further analysis of the DQF-COSY spectrum (Fig. 6), revealed a dispersion of H-4 resonances (δ 3.61–3.69), observable also in the ^{13}C NMR spectrum, where three peaks with approximate intensity ratio 1:4:1 appear at δ 83.1, 83.7 and 84.0, respectively. A COSY cross-peak showed the connection between H-4 and the broad H-5 resonance (δ 3.92), one-bond correlated with the carbon peaks at δ 74.4 and 74.7. Due to the inequivalence of the methylene protons at position 6, there is a further complication in the assignment of these proton resonances, that appear in the range δ 3.84–4.02, with a multiplet out of the crowd at δ 3.86; the related carbons (HMQC) give rise only to two peaks at δ 63.3 and 62.9, with approximate intensity ratio 1:5, respectively.

The above cited data suggest some considerations about the effect of the substitution of the β-CD structure with the cyclodipeptide moiety. The proton signals due to the ring A are remarkably influenced by the presence of the substituent; H-1, H-2, H-3 and H-5 appear at fields lower than the corresponding protons of the B–G rings. Contrary to this, H-4 experiences a shift to higher field; the methylene protons in position 6 also resonate at higher field due to the effect of the NH function; their chemical shift values differ markedly from that in unsubstituted β-CD ($\Delta\delta$ = 0.23 ppm instead of *ca.* 0.03 ppm) which suggests a somewhat rigid conformation for this part of the structure. In addition, it is worth noting that the ^1H $\Delta\delta$ (A − B–G) observed for the 'inner' protons, H-3 and H-5 are greater than those of the 'outer' protons H-1, H-2, and, to a lesser extent, H-4; this would suggest that the peptide 'pendant' could lie close to the β-CD cavity. A further observation concerns the 'dispersion' of ^{13}C chemical shifts due to the B–G glucopyranose rings: indeed, the $\Delta\delta$ observed for C-4 carbons is larger than that registered for the other carbons, and this can be correlated with a distortion of one of the torsion angles involved in the glycosidic linkage.[26]

These observations, prompted us to acquire more NMR data about the preferred conformation of **3**, with the aim to use this data as a reference for future studies about inclusion complexes of this molecule. Two ROESY[27] experiments were carried out at 500 MHz, using different mixing times. In addition, a sample of **3** was prepared in 90% H_2O solution, in order to provide evidence of possible dipolar interactions involving D_2O-exchangeable protons.

As far as the cyclopeptide moiety is concerned, a clear cross-correlation appeared in all the examined ROESY spectra between α- and β′-methylenes, while no NOE interaction has been observed between H-3′ and H-6′. This observation suggests that in **3**, the dioxopiperazine ring assumes preferentially the 'flagpole-boat' conformation, in agreement with previously reported data on c-(Glu-Glu) in aqueous solution.[28] The spectrum in 90% H_2O, showed a dipolar interaction between an NH signal at δ 8.15 and the β′-methylene signal; this NH proton has been assigned to the amide function adjacent to the ε-CH_2 on the basis of the COSY spectrum carried out on the same sample. These dipolar interactions

‡ The J values in Hz are: $^3J_{1,2}$ 3.2; $^3J_{2,3}$ 9.9; $^3J_{3,4}$ 9.5; $^3J_{4,5}$ 9.0; $^3J_{5,6}$ 8.5; $^3J_{5,6'}$ 2.0; $^2J_{6,6'}$ 13.0.

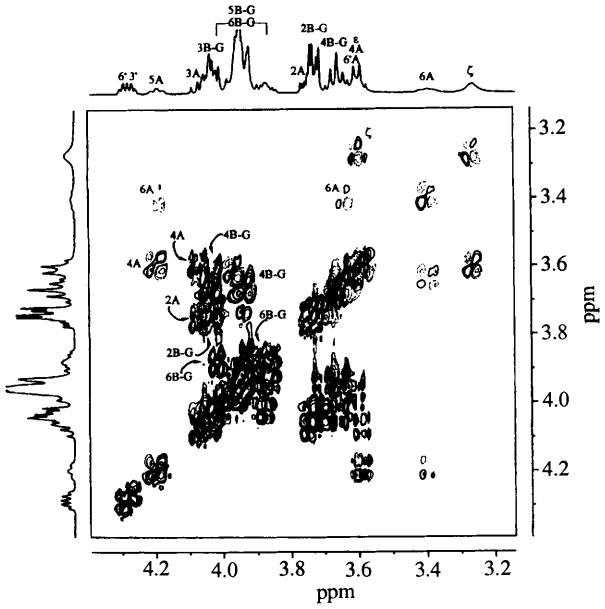


Fig. 6 Partial 500 MHz DQF-COSY spectrum of **3** in D_2O

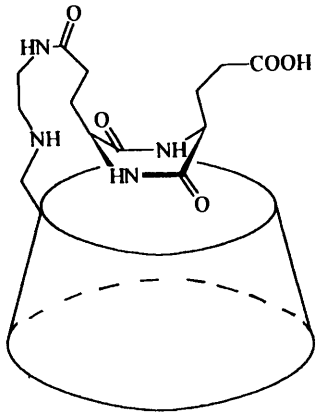


Fig. 7 Schematic spatial representation of **3**

reinforce the hypothesis, that a somewhat folded conformation is preferentially assumed by the pendant. Nevertheless, the ROESY spectra of **3** are poor because of cross-peaks between the substituent and the β-CD moiety: an exception is the observed NOE cross-peak between the multiplet assigned to the H-3′ and H-6′ protons and the H-6 (B–G) signals. This evidence is in favour of a conformation where the dioxopiperazine ring lies over the β-CD cavity, and can justify somewhat, the signals due to position 6 at δ 3.86 (^{1}H) and 63.3 (^{13}C), attributable to a 'perturbed' $-CH_2OH$ group on the top of the β-CD torus. Further NOE cross-peaks in the ROESY spectra of **3**, are clearly due to the expected dipolar interactions between glucopyranosidic protons, *i.e.* the typical 1–4 interactions between contiguous rings.[29] A schematic spatial representation of compound **3** is reported in Fig. 7.

Conclusions

The detailed high field NMR study reported in this paper, has demonstrated the value of this technique in the elucidation of the structural features of the asymmetrical substituted cyclodextrin derivative β-CDen-c-(Glu-Glu). In spite of the little spectral dispersion shown by this system, in comparison with other derivatives bearing aromatic moieties, the combined use of more advanced NMR experiments, has made it possible to assign all the proton and carbon resonances of the functionalized glucopyranose ring of the β-cyclodextrin in addition to a complete spectroscopic assignment of the substituent. In our opinion, a full structural NMR characterization of chemically modified cyclodextrins is important if their binding properties and basic phenomena governing the inclusion process are to be investigated.

Experimental

General

The melting point was recorded on a Mel-Temp II (Laboratory devices, USA) and is uncorrected. The optical rotation was recorded on a Jasco DIP-370 digital polarimeter and is given in units of 10^{-1} deg cm^2 g^{-1}. Column chromatographies were performed on CM-Sephadex C-25 (40–120 μm, Pharmacia) and DEAE-Sephadex A-25 (40–120 μm, Pharmacia). TLC was performed on glass plates precoated with 0.2 mm silica gel 60 F_{254} (Merck).

Materials

6-Deoxy-6-(2-aminoethylamino)-β-cyclodextrin (β-CDen) (**1**) was prepared from the parent cyclodextrin as described elsewhere.[17] (3*S*,6*S*)-3,6-Bis(3-carboxyethyl)-2,5-dioxopiperazine [c-(Glu-Glu)] (**2**), was obtained from Bachem (Switzerland) and used as received. DMF peptide synthesis grade (Millipore) was used. 2-(1*H*-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate coupling reagent (TBTU) and 1-hydroxybenzotriazole (HOBT) were purchased from Millipore Corp. and Aldrich, respectively, and used as provided.

Synthesis of β-CDen-c-(Glu-Glu) (3)

β-CDen, (**1**) (0.718 g, 6.1 × 10^{-4} mol), was added to a solution in DMF (8 cm^3) of c-(Glu-Glu) (**2**) (0.15 g, 5.8 × 10^{-4} mol) in the presence of HOBT (0.093 g, 6.1 × 10^{-4} mol) and TBTU (0.196 g, 6.1 × 10^{-4} mol). The reaction mixture was stirred vigorously at room temperature overnight under a nitrogen stream. The solution was evaporated to dryness *in vacuo* at a temperature not exceeding 30 °C. The crude product was chromatographed on a CM-Sephadex C-25 (NH_4^+ form) column using water as the eluent.

The product was detected with anisaldehyde reagent.[30] The TLC analysis of the fractions containing the desired product revealed the presence of a minor UV visible contaminant which was found to be HOBT. The HOBT contaminant was eliminated by anion exchange chromatography on a DEAE-Sephadex A-25 (HCO_3^- form) column which was eluted with a linear gradient of NH_4HCO_3 (0–0.2 mol dm^{-3}). The fractions containing the pure desired product were combined, concentrated to dryness under vacuum at 40 °C, repeatedly dissolved in water and dried to let ammonium hydrogen carbonate decompose. The glassy residue obtained was dissolved in a minimum amount of water and poured into 700 cm^3 of acetone. The resulting precipitate was collected by filtration and dessicated under vacuum. The product **3** was obtained in good yield (0.496 g, 3.5 × 10^{-4} mol, 60%) and showed a single spot on TLC analysis (R_f 0.41 eluent PrOH–H_2O–EtOAc–NH_3, 5:3:1:2). Compound **3** was dried under vacuum at 40 °C in the presence of P_2O_5, mp 255 °C (decomp.); $[\alpha]_D^{25}$ +106.9 (*c* 0.5 in H_2O); FAB–MS *m/z* 1418 (M + H^+); δ_H(250 MHz [$^2H_6$]DMSO, the chemical shift values refer to the residual DMSO peak assigned at δ 2.5) β-CD: 2.83 (m, 2 H, H-6A, H-6′A), 3.34 (m, 14 H, H-2, H-4), 3.59 (m, 26 H, H-3, H-6, H-5), 4.46 (br m, 6-OH), 4.82 (d, 7 H, H-1), 5.70 (br m, 14 H, 2-OH, 3-OH); substituent: 1.89 (m, 4 H, α-CH_2, α′-CH_2), 2.16 (m, 2 H, β-CH_2), 2.28 (m, 2 H, β′-CH_2), 2.59 (m, 2 H, ζ-CH_2), 3.12 (m, 2 H, ε-CH_2), 3.86 (m, 2 H, H-3′, H-6′), 7.84 (br t, 1 H, δ-NH), 8.15, 8.21 (br s, 1 H each, H-1′ or H-4′) (Found: C, 43.50; H, 6.52; N, 3.78. Calc. for $C_{54}H_{88}N_4O_{39}$·$4H_2O$: C, 43.55; H, 6.50; N, 3.76%).

NMR Spectroscopy

Preliminary ^{1}H NMR experiments were run on a 5.3 mmol dm^{-3} solution of **3** in [$^2H_6$]DMSO at 250.13 MHz on a Bruker

AC-250 instrument. High field NMR studies were performed on three different samples of **3** used for: (*a*) heteronuclear correlation experiments (35 mmol dm^{-3} solution in D_2O); (*b*) homonuclear correlation experiments (5.3 mmol dm^{-3} in D_2O); (*c*) ROESY experiment (5.3 mmol dm^{-3} in 90% H_2O, 10% D_2O). Samples (*a*) and (*b*) were freeze-dried from D_2O and final solutions were prepared just before use. The experiments were run at a constant temperature of 301 K. The chemical shift values refer to the residual water peak, assigned at δ 4.8. *J* Values are given in Hz. 1D ^1H NMR spectra at 750 MHz were recorded on a Bruker Avance DMX-750 spectrometer operating at 750.13 MHz. 1D ^{13}C NMR spectra (CPD and DEPT) were run on a Bruker Avance DRX-500 spectrometer operating at 125.69 MHz and were indirectly referenced to tetramethylsilane (TMS). Further one- and two-dimensional NMR experiments were carried out on Bruker Avance DMX-600 and DRX-500 spectrometers operating at 599.87 MHz and 499.87 MHz, respectively; some experiments were run on a Varian Unity-plus spectrometer operating at 499.87 MHz. All the experiments were performed with software supplied by the manufacturers. A 'reverse-detection' 5 mm probe was used. Homonuclear 2D spectra were typically acquired with 2 K data points for 512 increments of t_1 (zero filled to 1024 points), recording 8 transients for each increment with a recycle delay of 2 s. Double quantum filtered COSY (DQF-COSY) and ROESY spectra were acquired using the phase sensitive mode with time-proportional phase incrementation (TPPI).[31] 2D TOCSY spectra were acquired using a 10 kHz spin locking field with a MLEV-17 sequence of 80 ms and a mixing time of 80 ms. 1D TOCSY spectra were performed using a 80 ms Gaussian soft pulse and an array of mixing times from 5 ms to 105 ms. ROESY spectra were acquired with 2.5 kHz spin locking field using two different mixing times: 150 ms and 250 ms. Inverse detection proton–carbon correlation experiments, HMQC and HMBC were carried out with GARP[32] decoupling, acquiring 2 K data points for 256 increments of t_1 (zero filled to 512 points), with 16 (HMQC) or 32 (HMBC) transients for each increment.

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