TOAC: a useful C^{α}-tetrasubstituted α-amino acid for peptide conformational analysis by CD spectroscopy in the visible region. Part I

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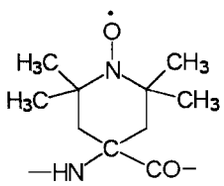
Doubly labelled 4-amino-4-carboxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TOAC)-containing trichogin analogues showed a correlation between the CD intensity of the TOAC transition and their conformation. The helical-inducing property of the TOAC residue is position dependent and, apart from the N-terminal position, better than that of Aib.

Introduction

Peptide labelling by coupling the achiral, C^{α}-tetrasubstituted α-amino acid 4-amino-4-carboxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TOAC) to the peptide N-terminus was introduced



TOAC

to use EPR to investigate the secondary structure of the peptide hormones angiotensin II[1] and α-MSH (α-melanocyte stimulating hormone).[2] This approach has been extended to other positions in the sequence and to other peptides in order to analyse the secondary structure[3–9] and the interaction between resin-bound chains during solid-phase peptide synthesis.[10] Other interesting features of TOAC, such as its fluorescence quenching capability and electrochemical properties,[4,11] have been explored. It is also known that TOAC, as are most of the conformationally constrained C^{α}-tetrasubstituted α-amino acids, is a strong turn and helix inducer.[4,6,9,11,12] Double TOAC labelled hexapeptides have been shown to fold in a 3_{10}-helix conformation in MeOH and to exist in an unordered structure in 1,1,1,3,3,3-hexafluoropropan-2-ol (HFIP) by EPR spectroscopy.[6] A preliminary CD investigation in the backbone region (peptide absorption) revealed an unusual CD spectrum that did not correspond to known elements of secondary structure such as α-helix, 3_{10}-helix, β-strand or unordered structures. The unusual CD profile has been ascribed to a dominating contribution by the induced CD of the achiral TOAC residues that precluded any unambiguous secondary structure assignment.[13] However, a differential intensity of the CD associated with the n→π* transition of the aminoxyl group (NO) of the TOAC residue as a function of solvent (Fig. 1) was seen as a possible correlation between the peptide conformation and the intensity of the NO CD transition.

To reduce the significance of the alleged TOAC CD contributions in the backbone region and to assess the validity of the correlation we investigated longer peptides. Analogues of the undecapeptide membrane-active, lipopeptaibol antibiotic [Leu-OMe 11] trichogin[14] (Tric-OMe) were obtained by replacing Aib (α-aminoisobutyric acid) with TOAC residues at positions 1 and 4, 1 and 8, 4 and 8, 1, 4 and 8 respectively (Table 1). Tric-OMe and an unrelated peptide (vsv-C) were also studied as reference peptides.

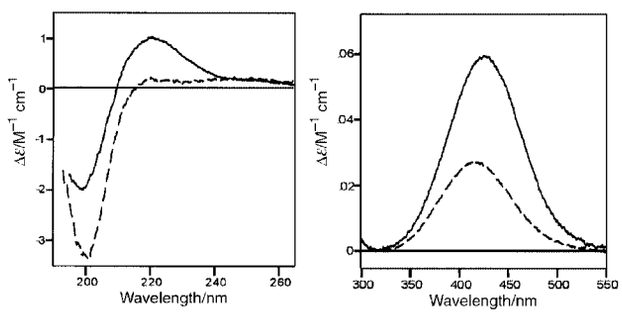


Fig. 1 CD spectra of Hex1.4 as a function of solvent: (——) MeOH and (– – – –) HFIP. Left: peptide region. Right: TOAC aminoxyl n→π* transition.

Results and discussion

Solvent effect

In the visible region the double TOAC Hex1.4 peptide shows a positive dichroism, with a maximum at about 430 nm in MeOH and about 420 nm in HFIP, associated with the TOAC aminoxyl n→π* transition (Fig. 1, right). However, the CD intensity of this transition in MeOH is about twice that in HFIP. As stated above, in the far-UV region the CD spectra of Hex1.4 in MeOH and HFIP (Fig. 1, left) are qualitatively different and do not resemble any known element of peptide secondary structure. The TOAC aminoxyl π→π* transition appears to dominate the peptide backbone region precluding any identification of the type of conformation adopted by the hexamer in solution.

This is not the case, however, for double TOAC-containing longer peptides, such as the analogues of the undecapeptide Tric-OMe (Table 1), where the far-UV region, associated with peptide secondary structure, is not dominated by TOAC CD contributions. In MeOH Tric4.8 exhibits two intense negative

Table 1 Amino acid sequences of the peptides studied in this work

Peptide	Abbreviation
Boc-***T***$_1$-A-***T***$_3$-A-A-A-O*t*Bu	Hex1.3
Boc-***T***$_1$-A-A-***T***$_4$-A-A-O*t*Bu	Hex1.4
*n*Oct-***B***$_1$-G-L-***B***$_4$-G-G-L-***B***$_8$-G-I-L-OMe	Tric-OMe
*n*Oct-***T***$_1$-G-L-***T***$_4$-G-G-L-***B***$_8$-G-I-L-OMe	Tric1.4
*n*Oct-***T***$_1$-G-L-***B***$_4$-G-G-L-***T***$_8$-G-I-L-OMe	Tric1.8
*n*Oct-***B***$_1$-G-L-***T***$_4$-G-G-L-***T***$_8$-G-I-L-OMe	Tric4.8
*n*Oct-***T***$_1$-G-L-***B***$_4$-G-G-L-***B***$_8$-G-I-L-OMe	Tric1
*n*Oct-***B***$_1$-G-L-***T***$_4$-G-G-L-***B***$_8$-G-I-L-OMe	Tric4
*n*Oct-***B***$_1$-G-L-***B***$_4$-G-G-L-***T***$_8$-G-I-L-OMe	Tric8
K-L-I-G-V-L-S-S-L-F-R-P-K	vsv-C

T, TOAC. ***B***, Aib.

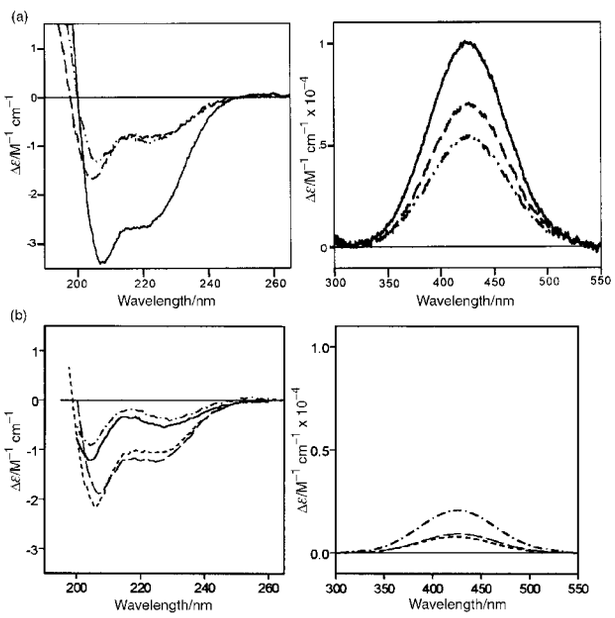


Fig. 2 (a) CD spectra of Tric4.8 (———), Tric1.4 (— — —) and Tric1.8 (— ·· —) in MeOH. Left: peptide region. Right: TOAC aminoxyl n→π* transition and their Δε expressed in terms of per TOAC residue. (b) CD spectra of Tric-OMe (———), Tric1 (— · —), Tric4 (— — —) and Tric 8 (– – – –) in MeOH. Left: peptide region. Right: TOAC aminoxyl n→π* transition and their Δε expressed in terms of per TOAC residue.

CD bands at 220 and 208 nm, followed by a positive band below 200 nm, characteristic of helical conformations (Fig. 2).

Tric1.4, Tric1.8 and Tric-OMe give signals of remarkably lower intensities as compared to Tric4.8, thus indicating the latter has a significantly higher content of helical conformation.

The estimation of the α-helical content of the far-UV CD data[15] reveals that Tric4.8 is the most helical peptide (33.0%) in MeOH followed by Tric8 (14.0%), Tric4 (11.1%), Tric1.4 (10.8%), Tric1.8 (9.0%) and Tric1 (3.2%) and that the parent peptide Tric-OMe has only 5% of helical content (Table 2). However, the decreased content of helical conformation is accompanied by a decreased intensity of the TOAC n→π* transition in the visible (400–450 nm) region (Fig. 2) only for the double TOAC peptides. For the mono TOAC peptides the correlation does not hold. In terms of CD intensity of the NO transition, the value for Tric1.8 is about three-fold that of Tric1 and about six-fold those of Tric4 and Tric8, despite the higher helical folding of both Tric4 and Tric8 than Tric1 (Table 2). The rationale of the aminoxyl CD data is that the increased CD intensity of double TOAC peptides is due to a non-exciton coupling between two TOAC residues, which is further enhanced when two vicinal TOAC residues are folded in an helical conformation.

It is important to note that the helix-inducing effect of the TOAC residue is position dependent with the highest helical

Table 2 Estimation of α-helix content in MeOH from CD data

Peptide	*T*/°C	α-Helix (%)
Tric4.8	23	33.0
	5	38.4
	−28	41.1
	−47	35.6
	−79	28.1
	−92	17.7
Tric4	23	14.0
Tric8	23	11.1
Tric1.4	23	10.8
Tric1.8	23	9.0
Tric1	23	3.2
Tric-OMe	23	5.0
vsv-C	23	13.1

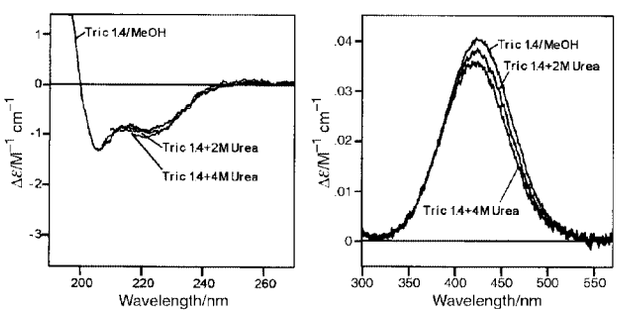


Fig. 3 CD spectra of Tric1.4 in MeOH as a function of urea concentration. Left: peptide region. Right: TOAC aminoxyl n→π* transition.

content observed for Tric4.8 and the lowest for Tric1. The TOAC substitution at position 1 appears to destabilise the amount of helical conformation when compared to that of Tric-OMe. This is consistent with a similar reduction in percentage of the helical content estimated for both Tric1.4 and Tric1.8 when compared to those of Tric4 and Tric8 respectively (Table 2). This observation is in agreement with the EPR data, which indicate that the TOAC residue is a less effective helix inducer at the N-terminal position.[8,9] An important implication is that, apart from the N-terminal position, the TOAC residue has a better helical-inducing effect than the Aib residue.

If the CD intensity of the TOAC n→π* transition is correlated to the helical content of TOAC-containing peptides, then a perturbation of the conformation of the double TOAC-containing trichogin analogues should affect the intensity of the TOAC aminoxyl n→π* CD band. Urea and cryogenic denaturations were therefore carried out to test the aminoxyl CD intensity–peptide conformation correlation.

Urea denaturation

An unrelated TOAC-free vsv-C peptide (KLIGVLSSLFRPK), used as a control peptide, is readily denatured in 4 M urea (CD spectrum not shown). Surprisingly, all double TOAC-containing Tric-OMe analogues show a remarkable stability towards urea denaturation with very small CD changes observed in both far-UV and visible regions that are still consistent with the proposed correlation. As an example, Fig. 3 illustrates the behaviour of Tric1.4.

Cryogenic studies

At room temperature a peptide is usually present in a conformational equilibrium that is temperature dependent. A cryogenic study is a useful experiment to monitor thermal stability and conformational change. This is readily seen for the TOAC-free vsv-C peptide in MeOH, where a helix-extended conformation transition takes place on lowering the temperature (Fig. 4). At −79 °C the CD spectrum reveals a dominating content of extended (polyproline type II) conformation characterised by a

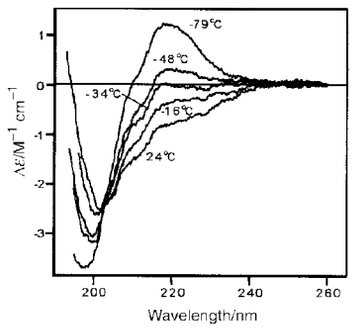


Fig. 4 CD spectra of the peptide region of vsv-C in MeOH as a function of temperature.

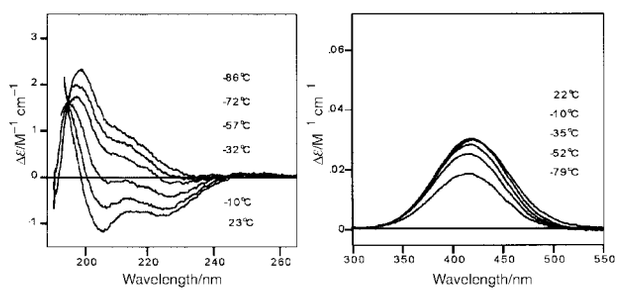


Fig. 5 CD spectra of Tric1.4 in MeOH as a function of temperature. Left: backbone region. Right: TOAC aminoxyl n→π* transition.

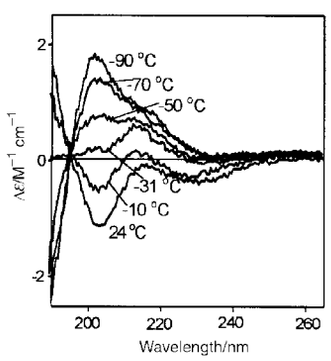


Fig. 6 CD spectra of Tric-OMe in MeOH as a function of temperature.

positive CD band at about 218 nm.[16] For Tric1.4, a decreased TOAC n→π* CD intensity is accompanied in the backbone region by a decreased content of helical conformation (Fig. 5). A similar behaviour is also observed in the peptide region of Tric-OMe on lowering the temperature (Fig. 6). Since Tric-OMe lacks any TOAC residue, the almost identical cryogenic behaviour of Tric1.4 and Tric-OMe (Fig. 7, left) rules out any significant TOAC CD contribution in the peptide region. On this basis the CD spectrum of Tric1.4 at low temperature has to be ascribed to an ordered backbone structure of a likely turn type,[17] though its geometry has not yet been identified. The presence of an isodichroic point for both Tric1.4 (Fig. 5, left) and Tric-OMe (Fig. 6) is indicative of an equilibrium mixture between two species.

For Tric4.8 an increased CD intensity in the visible region from 22 to −20 °C, followed by a decreased intensity from −20 to −77 °C (Figs. 7 and 8), is accompanied in the peptide region by a similar trend that corresponds respectively to an increased and decreased helical content. Tric1.4 shows similar trends in the peptide and TOAC n→π* regions (Fig. 5), which are however different from those exhibited by Tric4.8. This rules out the solvent effect as the main factor responsible for the CD intensity changes of the TOAC n→π* transition as a function of temperature. The helix-turn conformation transition of the

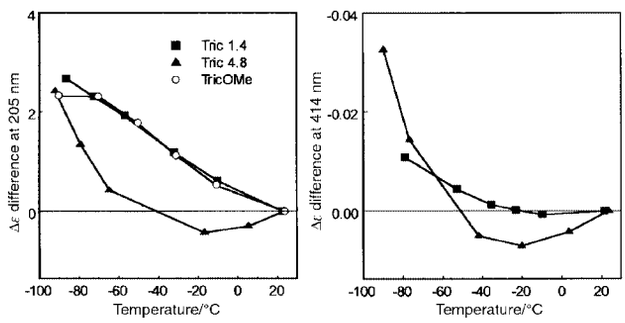


Fig. 7 Plot of $\Delta\varepsilon$ values *versus* temperature of Tric1.4, Tric4.8 and Tric-OMe in MeOH. Left: $\Delta\varepsilon$ values at 205 nm. Right: $\Delta\varepsilon$ values at 414 nm. (All the CD values at 205 and 414 nm as a function of temperature have had the CD values at 20 °C subtracted from them in order to offset the plots to the same origin.)

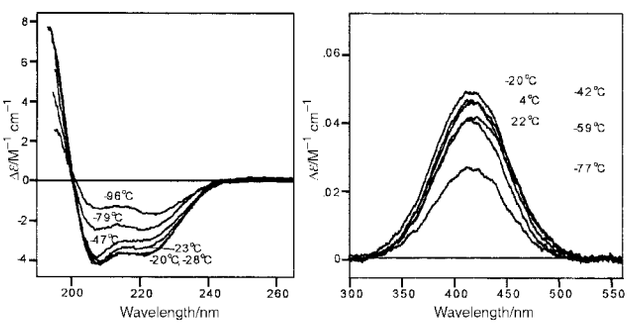


Fig. 8 CD spectra of Tric4.8 in MeOH as a function of temperature. Left: backbone region. Right: TOAC aminoxyl n→π* transitions.

double TOAC-containing undecapeptides, monitored under cryogenic conditions in both far-UV and visible regions, provides supportive evidence for the usefulness of the TOAC n→π* transition intensity as a criterion to identify the presence of helical conformations in double TOAC-containing short peptides. Based on this criterion, the CD intensity of the TOAC n→π* transition is enhanced on increasing the peptide helical content. Applied to double TOAC labelled hexapeptides Hex1.3 and Hex1.4, a differential temperature dependence of the TOAC n→π* CD band is therefore associated with differential conformational behaviours. For Hex1.3, the TOAC n→π* CD band increases from 23 to −49 °C and decreases from −49 to −89 °C, whilst for Hex1.4 the n→π* band increases from 23 to −71 °C (data not shown). At low temperature, Hex1.4 appears therefore to be more helical and structured than Hex1.3, whilst at room temperature both peptides appear to be similarly folded. The cryogenic CD study is additional supportive evidence of the relationship between the intensity of the TOAC aminoxyl n→π* transition and the helical conformation in solution.

Conclusions

A conformational analysis as a function of urea concentration and temperature has provided evidence for a correlation between the CD intensity of the TOAC aminoxyl n→π* transition of double TOAC labelled [Leu-OMe 11] trichogin analogues and their conformations. The reason why the α-helix correlation is not quantitative is because the CD of the aminoxyl chromophore of the TOAC residues, like the CD of the amide chromophore of a backbone peptide, is conformation dependent. Other conformations such as β-turns, 3_{10} helix, extended and irregular structures contribute to the CD of the TOAC residues.

From this work, the CD intensity of the TOAC aminoxyl group can be used as a criterion to assess qualitatively the presence of helical conformations in double TOAC-containing peptides. This will allow conformational information to be

extracted even in cases where the use of non-transparent far-UV solvents, aromatic protecting groups or aromatic amino acid rich peptides would normally prevent any conformational analysis from being carried out. Without being too bulky, the TOAC residue would provide a useful CD probe to monitor conformational changes of transmembrane and antimicrobial peptides imbedded in phospholipid bilayers or membranes, as well as to study the rules that govern the initiation and propagation of helical conformations in peptides.

Another important observation from this work is that the helical-inducing property of the TOAC residue is position dependent and, apart from the N-terminal position, better than that of the Aib residue.

Experimental

Materials

The solution synthesis and characterization of Hex1.3 and Hex1.4,[6] Tric-OMe,[18] and Tric1.4, Tric1.8, Tric4.8, Tric1, Tric4 and Tric8[9a] have been reported elsewhere. The vsv-C peptide was prepared by solid-phase synthesis.[16] HFIP and spectrosol MeOH were purchased from Aldrich and BDH, respectively.

Circular dichroism

CD spectra were recorded with Jasco J720 and J600 spectropolarimeters flushed with evaporated nitrogen to improve performance below 200 nm. A solution concentration of 0.2 mg ml^{-1} and 0.1 cm cell pathlength were used in the far-UV CD region, whereas a concentration of 2 mg ml^{-1} and 2 cm cell pathlength were used in the near-UV CD region. Apart from Fig. 2a and 2b in the visible region, all spectra were reported in terms of $\Delta\varepsilon = \varepsilon_L - \varepsilon_R$ (M^{-1} cm^{-1}) using a mean molecular weight (MW) per amino acid residue (peptide MW divided by the number of amino acid residues). For Fig. 2a and b in the visible region, we used the MW fraction calculated as the MW of the TOAC residues divided by the peptide MW in order to compare the CD spectra as a function of TOAC residue fraction. The cryogenic studies were carried out using a Jouan attachment. The estimation of secondary structure content was determined following Malik's method[15] using a principal component regression analysis (Grams/32 suite program, Galactic Industries Corporation) with a calibration data set of 16 proteins obtained from Hennessey and Johnson.[19]

References

1 (*a*) C. R. Nakaie, G. Groissis, S. Schreier and A. C. M. Paiva, *Braz. J. Med. Biol. Res.*, 1981, **14**, 173; (*b*) C. R. Nakaie, S. Schreier and A. C. M. Paiva, *Biochim. Biophys. Acta*, 1983, **742**, 63.
2 S. R. Barbosa, E. M. Cilli, M. T. Lamy-Freund, A. M. L. Castrucci and C. R. Nakaie, *FEBS Lett.*, 1999, **446**, 45.
3 R. Marchetto, S. Schreier and C. R. Nakaie, *J. Am. Chem. Soc.*, 1993, **115**, 11042.
4 C. Toniolo, E. Valente, F. Formaggio, M. Crisma, G. Pilloni, G. Corvaja, A. Toffoletti, G. Martinez, M. P. Hanson, G. L. Millhauser, C. George and J. L. Flippen-Anderson, *J. Pept. Sci.*, 1995, **1**, 45.
5 M. L. Smythe, C. R. Nakaie and G. R. Marshall, *J. Am. Chem. Soc.*, 1995, **117**, 10555.
6 P. Hanson, G. Millhauser, F. Formaggio, M. Crisma and C. Toniolo, *J. Am. Chem. Soc.*, 1996, **118**, 7618.
7 (*a*) P. Hanson, G. Martinez, G. Millhauser, F. Formaggio, M. Crisma, C. Toniolo and C. Vita, *J. Am. Chem. Soc.*, 1996, **118**, 271; (*b*) P. Hanson, D. J. Anderson, G. Martinez, G. Millhauser, F. Formaggio, M. Crisma, C. Toniolo and C. Vita, *Mol. Phys.*, 1998, **95**, 957.
8 Y. V. Toropov, S. A. Dzuba, Y. D. Tsvetkov, V. Monaco, F. Formaggio, M. Crisma, C. Toniolo and J. Raap, *Appl. Magn. Reson.*, 1998, **15**, 237.
9 (*a*) V. Monaco, F. Formaggio, M. Crisma, C. Toniolo, P. Hanson, G. Millhauser, C. George, J. R. Deschamps and J. L. Flippen-Anderson, *Bioorg. Med. Chem.*, 1999, **7**, 119; (*b*) V. Monaco, F. Formaggio, M. Crisma, C. Toniolo, P. Hanson and G. L. Milhauser, *Biopolymers*, 1999, **50**, 239; (*c*) D. J. Anderson, P. Hanson, J. McNulty, G. L. Milhauser, V. Monaco, F. Formaggio, M. Crisma and C. Toniolo, *J. Am. Chem. Soc.*, 1999, **121**, 6919.
10 E. M. Cilli, R. Marchetto, S. Schreier and C. R. Nakaie, *Tetrahedron Lett.*, 1997, **38**, 517.
11 C. Toniolo, M. Crisma and F. Formaggio, *Biopolymers* (*Pept. Sci.*), 1998, **47**, 153.
12 M. Crisma, V. Monaco, F. Formaggio, C. Toniolo, C. George and J. L. Flippen-Anderson, *Lett. Pept. Sci.*, 1997, **4**, 213.
13 T. T. Bui, C. Toniolo, M. Crisma, F. Formaggio, R. Hussain and G. Siligardi, in *Peptides 1998*, S. Bajusz and F. Hudecz, eds., Akadémiai Kiadó, Budapest, 1999, pp. 348–349.
14 (*a*) C. Auvin-Guette, S. Rebuffat, Y. Prigent and B. Bodo, *J. Am. Chem. Soc.*, 1992, **114**, 2170; (*b*) C. Toniolo, C. Peggion, M. Crisma, F. Formaggio, X. Shui and D. S. Eggleston, *Nature: Struct. Biol.*, 1994, **1**, 908.
15 K. M. Malik, *PhD Thesis*, 1997, University of London.
16 G. Siligardi and A. F. Drake, *Biopolymers* (*Pept. Sci.*), 1995, **37**, 281.
17 R. W. Woody, in *The Peptides*; V. J. Hruby, ed., Academic Press, New York, 1985; pp. 15–113.
18 C. Toniolo, M. Crisma, F. Formaggio, C. Peggion, V. Monaco, C. Goulard, S. Rebuffat and B. Bodo, *J. Am. Chem. Soc.*, 1996, **118**, 4952.
19 J. P. Hennessey and W. C. Johnson, Jr., *Biochemistry*, 1981, **20**, 1085.