Aggregation of a tetrapeptide derivative [Boc-Ile-Gly-Met-Thr(Bzl)-OBzl] in chloroform

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The tetrapeptide derivative Boc-Ile-Gly-Met-Thr(Bzl)-OBzl 1 forms micellar aggregates in chloroform. Evidence for micelle formation in chloroform has been obtained by IR, Raman scattering, fluorescence spectroscopic methods and calcium picrate extraction techniques. The thermodynamic data indicate that the driving force for the micellisation is enthalpic in nature.

Introduction

One of the most interesting areas of organic synthesis involves making organic molecules which can mimic biological macromolecules with structures such as proteins and nucleic acids. Although these molecules are largely covalently bonded, their ultimate structure is dictated by non-covalent forces. In fact, the functions of these molecules are determined by their self-assembled states which in turn depend on the non-covalent interactions. The importance of these non-covalent forces in designing novel synthetic molecules provides a compelling motivation to study this phenomenon in detail.[1] Peptide modules offer an interesting model to investigate the molecular forces which are involved in the self assembly of complex biological systems. Self assembling peptides are known to form various types of ordered aggregates such as micelles,[2–4] monolayers,[5] membranes[6] and nanotubules[7,8] in aqueous and non-aqueous solutions.

There are two types of interaction that stabilise these assemblies, namely, the solvophobic interaction and the packing component, the latter being the interaction between solvophobic groups in the interior of the aggregate. A knowledge of the nature and magnitude of the energetics of these interactions in organised aggregates is needed to understand their respective roles in the formation of an assembly.[8] For the past two decades extensive work has been carried out on the micellisation of amphiphiles in aqueous solution. However, only a few reports are available on micellisation in apolar media.[9] The ordered aggregates of peptide derivatives in an apolar medium like cyclohexane and benzene have been reported by Ihara *et al*.[10] We have already demonstrated that the tripeptide Boc-Val-Val-Ile-OMe, found in the parallel β-sheet region of triosephophase isomerase, forms micelles in chloroform. Evidence for this was obtained using UV–visible, fluorescence and NMR spectroscopic techniques.[2b] In apolar media, the driving force for aggregate formation is attributed to the high solvophobic property of the −NHC(=O)− groups.[10] The strong directional nature of the amide groups, combined with their ability to form hydrogen bonds, may result in interesting entropy–enthalpic interactions. Therefore, a search for the peptide components containing solvophobic amide groups which can form persistent packing motifs is expected to be worthwhile.[11,12] These models will also be useful in exploring the contribution of hydrogen bonding and solvophobic interactions to the free energy change involved in the self assembly of peptides.[13]

We report here that the tetrapeptide Boc-Ile-Gly-Met-Thr(Bzl)-OBzl **1** with four amide groups can self assemble in chloroform to form a stable micelle at various temperatures. The sequence of the peptide **1** was found in the β-structure region of proteinase of HIV-1 (RP 93-96).[14] The micelle formation of the above tetrapeptide is analysed in terms of the thermodynamic functions. The extraction of calcium picrate by molecules containing multiple polar groups like cyclosporin,[15] calixarenes[16,17] and crown ethers[18] are reported in the literature. Here we are also using the calcium picrate extracting property of the peptide in chloroform for the determination of the critical micellar concentration (c.m.c.).

Experimental

The tetrapeptide **1** was prepared by the solution phase method using dicyclohexylcarbodiimide–hydroxybenztriazole reagents.[19] The peptide was purified on silica gel using methanol–chloroform (1:9) as eluent. The ^{1}H NMR spectra were recorded using a Bruker MSL 300P (300 MHz) spectrometer. ^{1}H NMR ($CDCl_3$) δ 0.92 (d, δ-CH_3 Ile, 3H), 1.1 (m, γ_b-CH_2 Ile, 2H), 1.24 (s, γ_a-CH_3 Ile, 3H), 1.43 [s, $(CH_3)_3$C Boc, 9H], 1.63 (s, β-CH Ile, 1H), 2.03 (s, β-CH_2 Met, 2H), 2.07 (s, S-CH_3 Met, 3H), 2.59 (s, γ-CH_2 Met, 2H), 3.89 (d, α-CH Ile, 1H), 3.98 (m, α-CH_2- Gly, 2H), 4.18 (m, α-CH- Met, 1H), 4.65 (m, β-CH_3 Thr, 3H), 5.10 (s, COO-CH_2- Thr, 2H), 5.16 (s, C_6H_5- O-CH_2 Thr, 2H), 5.35 (s, NH Ile, 1H), 6.95 (s, NH Met, 1H), 7.1 (m, NH Thr, 1H), 7.25 (s, COO-CH_2-C_6H_5 Thr, 5H), 7.35 (s, O-CH_2-C_6H_5 Thr, 5H), 7.5 (s, NH Gly, 1H). HPLC grade chloroform was used for all the experiments. The intensity of the Raman scattering peak of chloroform was observed using a fluorescence spectrophotometer (Hitachi, model 650-40). The spectrum was obtained by excitation at 405 nm and was observed around 460 nm in chloroform.[20] As the concentration of the peptide increased, the intensity of the band at 460 nm increased and abrupt changes in the value of the initial slopes at a particular concentration were considered to be the c.m.c.[3] Pyrene (Aldrich) was recrystallised three times from ethanol. The ammonium salt of 8-anilino-1-naphthalenesulfonic acid (ANS) was obtained from Fluka and used as such.

The IR absorption spectra of peptide **1** at different concentrations in chloroform were recorded using the Nicolet 20DXB system. The accuracy of the band position is *ca*. ±0.1 cm^{-1}. The measurements were recorded at 22 °C and the value thus obtained was similar to the temperature at which the c.m.c. was obtained by the Raman scattering method (Table 1). The extraction of calcium picrate by a chloroform solution of peptide at various concentrations was studied as follows. The Tris-HCl (pH = 8.1) buffer solution containing calcium chloride (0.05 mol) and picric acid (0.54 mmol) was stirred with a magnetic stirrer for 1 h and allowed to stand for *ca*. 30 min. The

Table 1 Critical micelle concentration (c.m.c.) and other thermodynamic parameters of the tetrapeptide micelles at various temperatures

T/°C	c.m.c./mmol	$\Delta_m G°$/kJ mol^{-1}	$\Delta_m H°$/kJ mol^{-1}	$\Delta_m S°$/JK^{-1} mol^{-1}	$\Delta_p C°$/JK^{-1} mol^{-1}
3	1.50[a]	−14.85 ± 0.07	−40.07 ± 0.34	−91.38 ± 0.98	
	1.60[b]				
11	2.15[a]	−14.58 ± 0.08	−42.43 ± 0.37	−98.05 ± 1.57	
	2.00[b]				
16	3.00[a]	−13.92 ± 0.04	−44.44 ± 0.13	−103.86 ± 1.16	−301.65 ± 2.66
	3.10[b]				
20	4.25[a]	−13.38 ± 0.08	−45.16 ± 0.39	−108.03 ± 2.00	
	4.00[b]				
22	5.00[c]	−12.97 ± 0.02	−45.78 ± 0.39	−111.22 ± 1.25	
	5.10[b]				
25	6.00[a]	−12.64 ± 0.05	−46.71 ± 0.40	−114.35 ± 1.19	
	6.20[b]				

[a,b,c] c.m.c. determined by Raman scattering, ANS fluorescence and IR methods, respectively.

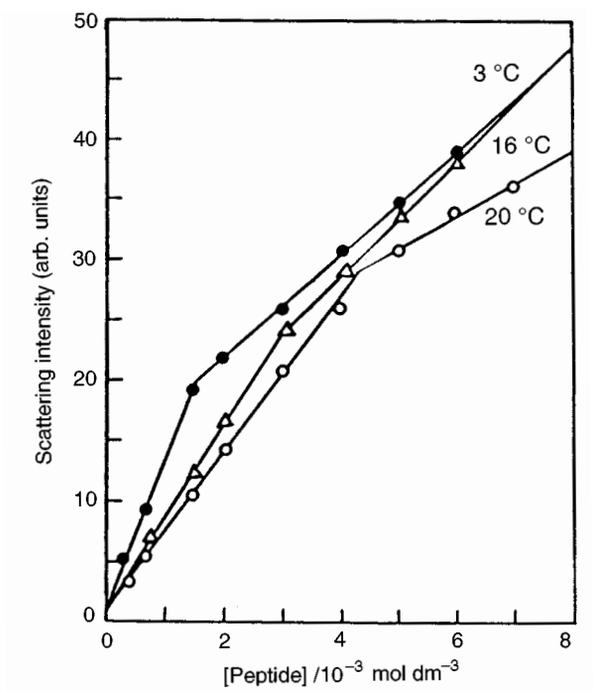

Fig. 1 Plot of Raman scattering intensity of chloroform solutions *vs.* peptide concentration at various temperatures. λ_{ex} = 405 nm, λ_{em} = 460 nm.

amount of calcium picrate distributed in the chloroform and in the buffer was determined by measuring its absorbance (λ_{max} = 356 nm) using a Hewlett-Packard diode-array 8452A spectrophotometer at 30 °C.

Results and discussion

The plots of Raman scattering intensity at 460 nm *vs.* peptide concentration at various temperatures are shown in Fig. 1 and the c.m.c. values are given in Table 1. The micelle formation was further confirmed from the fluorescence intensity of 8-anilino-1-naphthalenesulfonic acid at various peptide concentrations (Table 1).

The c.m.c. of the peptide was also obtained by calcium picrate extraction measurements. The absorbance of calcium picrate (λ_{max} = 356 nm) in water and chloroform layers was plotted *vs.* the peptide concentration to indicate a change in slope at 7 and 6.6 mmol, respectively, which is the c.m.c. of the peptide at 30 °C (Fig. 2). It should be noted that the extent of extraction in chloroform increases on increasing the peptide concentration. However, after reaching the c.m.c. the decrease in the slope of the plot of absorbance of calcium picrate *vs.* peptide concentration implies that the aggregated form of the peptide reduces the calcium picrate extraction in chloroform. The corresponding

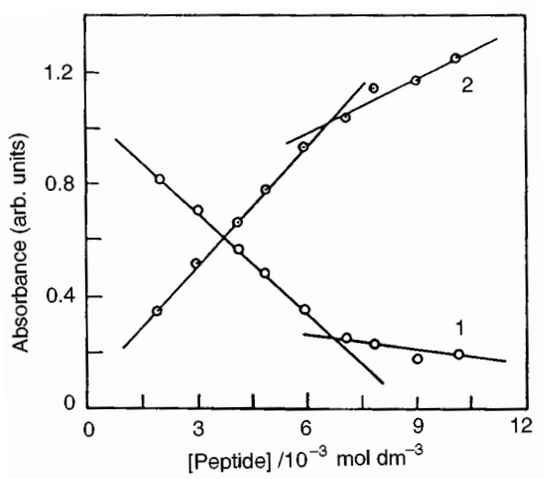

Fig. 2 Absorbance of calcium picrate in the buffer (1) and $CHCl_3$ (2) layer at various peptide concentrations

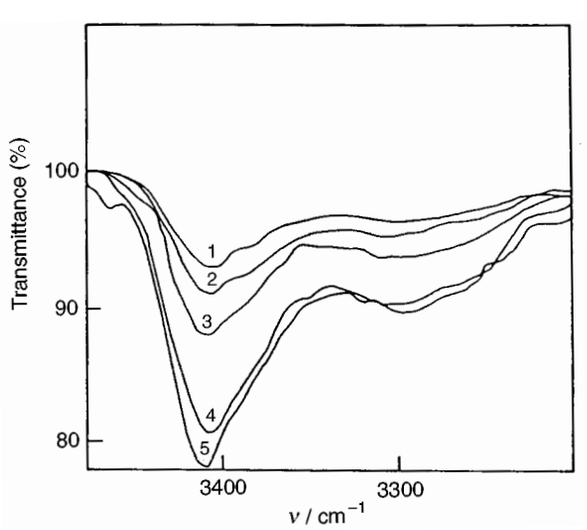

Fig. 3 Amide A region of the IR spectra of the peptide **1** at various concentrations. Curve nos. 1–5: 0.5, 2, 3, 5 and 7 mmol peptide in chloroform, respectively.

decrease in the concentration of calcium picrate in aqueous solution is shown in Fig. 2 (1).

The NH stretching region (amide A) in the IR spectra of the tetrapeptide shows two absorption peaks (Fig. 3). A peak around 3410 cm^{-1} was attributed to the presence of solvated −NH− stretching and the other peak around 3300 cm^{-1} is due to hydrogen bonded −NH− groups.[21,22] The amide A region of the tetrapeptide at various concentrations is depicted in Fig. 3. The relative molar absorption of the band near 3410 cm^{-1} was plotted *vs.* the concentration of the peptide **1**, showing an

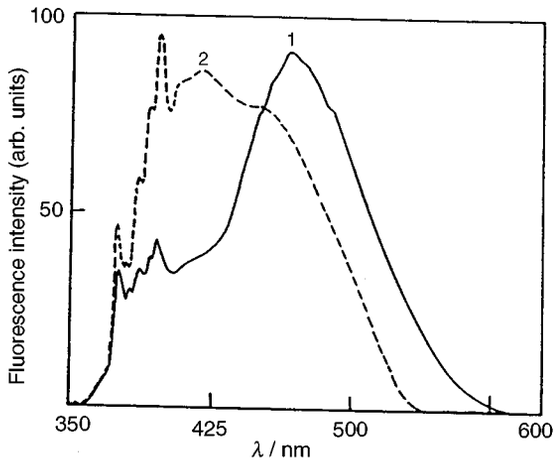

Fig. 4 Fluorescence spectra of pyrene in chloroform at pre- and post-micellar concentrations of peptide **1** (at 22 °C). [pyrene] = 1.3×10^{-5} mol (fixed). λ_{ex} = 335 nm, curve nos. 1–2: 1 and 8 mmol, respectively.

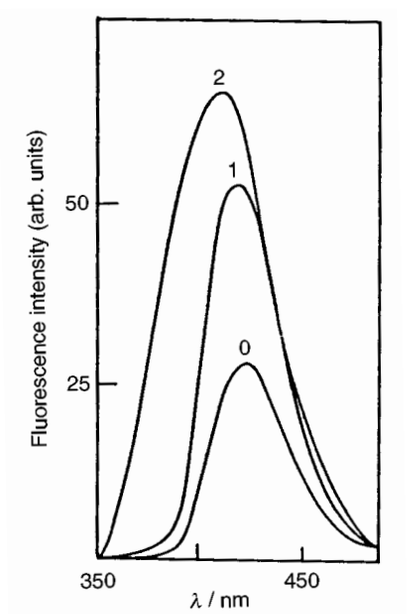

Fig. 5 Fluorescence spectra of ANS in chloroform at various concentrations of peptide **1** at 20 °C [ANS] = 1×10^{-5} mol (fixed). λ_{ex} = 346 nm, curve nos. 0–2; 0, 0.45 and 6 mmol, respectively.

abrupt change of slope at 5 mmol concentration (figure not shown). It is interesting to note that the broad band at 3300 cm^{-1} is present even in the pre-micellar state, suggesting the occurrence of intermolecular hydrogen bonding. After reaching the c.m.c. the relative molar absorption of the band which corresponds to weakly hydrogen bonded –NH– or solvated –NH– (3410 cm^{-1}) achieved a constant value with increasing concentration. This indicates that micellisation results in intermolecular hydrogen bonding between peptide and chloroform molecules.[2,23]

The c.m.c. values obtained by IR, Raman scattering intensity measurements at 460 nm and ANS fluorescence studies are in good agreement with each other. The c.m.c. of the tetrapeptide at various temperatures are given in Table 1.

Further studies on the nature of the aggregate were carried out using pyrene as a fluorescence probe. The fluorescence spectrum at a pre-micellar concentration consists of a structured part resulting from monomer pyrene emission and a structureless band at longer wavelength corresponding to excimer (excited dimer) pyrene emission (Fig. 4). The relatively high excimer fluorescence intensity indicates that a substantial number of pyrene molecule participate in excimer formation in the pre-micellar concentration. However, after attaining the c.m.c.

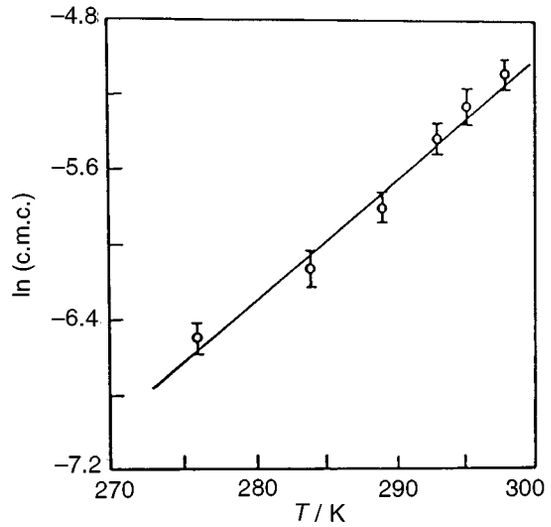

Fig. 6 Plot of ln (c.m.c.) *vs.* T

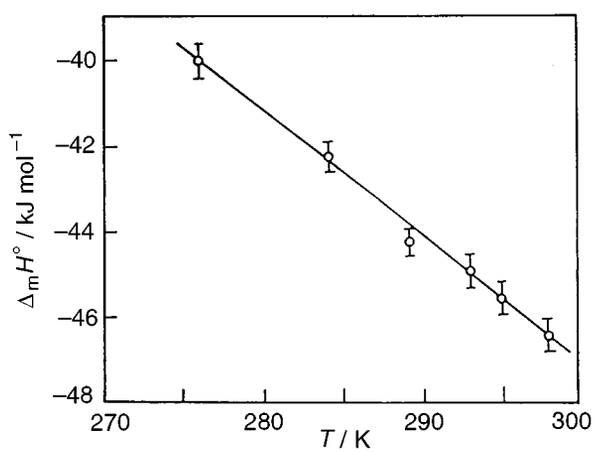

Fig. 7 Plot of $\Delta_m H^\circ$ *vs.* T

the excimer fluorescence intensity decreases indicating the peptide–pyrene interaction at post-micellar concentration. This decreases the possibility of encounter between the two probe molecules.[24]

The fluorescence changes of pyrene and ANS probes bound to peptides are indicative of the aggregate interior polarity.[24,25] The ratio of the intensity of the first vibronic peak to the third vibronic peak (I_1/I_3) of pyrene at the pre-micellar concentration is 1.02. This indicates that pyrene binds to the aromatic protecting groups of the peptide in the monomer state. At post-micellar concentration (7 mmol, 22 °C) the I_1/I_3 value is 0.80. This is indicative of pyrene binding to micellar regions containing alkyl groups. This preferential binding is ratified by ANS fluorescence studies which show an increase in the fluorescence intensity at pre- and post-micellar concentrations of the peptide. On micellisation, a small blue shift in the λ_{em} indicates increased microviscosity around ANS in peptide micelles in chloroform (Fig. 5).

Using a biphasic micellar model,[26] the standard Gibbs free energy change for micelle formation, $\Delta_m G^\circ$ of the peptide has been calculated using eqn. (1).

$$\Delta_m G^\circ = RT \ln (\text{c.m.c.}) \quad (1)$$

The standard enthalpy change for micelle formation ($\Delta_m H^\circ$) was calculated using the value obtained from the slope of the plot ln (c.m.c.) *vs.* temperature (Fig. 6). $\Delta_m H^\circ$ was calculated using eqn. (2).

$$\Delta_m H^\circ = -RT^2 [\mathrm{d} \ln (\text{c.m.c.})/\mathrm{d}T] \quad (2)$$

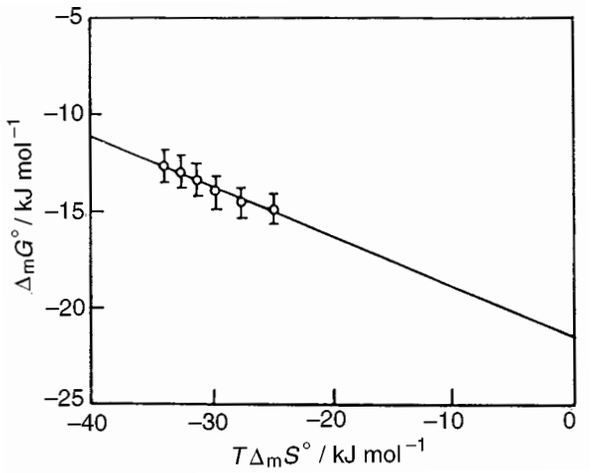

Fig. 8 Plot of $\Delta_m G^\circ$ *vs.* $T\Delta_m S^\circ$

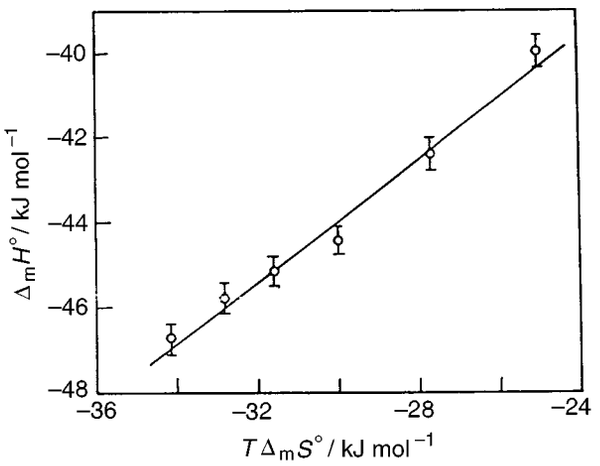

Fig. 9 Plot of $\Delta_m H^\circ$ *vs.* $T\Delta_m S^\circ$

The change in standard heat capacity at constant pressure ($\Delta_p C^\circ$) was calculated from the slope of the plot $\Delta_m H^\circ$ *vs.* temperature (Fig. 7).

$$\Delta_p C^\circ = (\partial \Delta_m H^\circ / \partial T) \quad (3)$$

The thermodynamic parameters thus calculated are tabulated in Table 1. From the values of negative $\Delta_m H^\circ$ and negative $\Delta_m S^\circ$, it is clear that the driving force for peptide aggregation is enthalpic in nature. When peptide aggregates, the solvophobic groups of peptide monomers (≻C=O and NH) are 'clustered' together, resulting in a fairly close approach of the ≻C=O groups. This results in electrostatic destabilisation due to unfavourable dipole–dipole interactions. However, such unfavourable interactions could be compensated by solvation of the amide bonds with chloroform molecules.[27]

The negative $\Delta_p C^\circ$ signifies the interaction of solvent with the peptidic moieties[28–31] and it should be noted that the solvated NHs increases on micellisation (Fig. 3). Table 1 shows that $\Delta_m S^\circ$ and $\Delta_m H^\circ$ values for the tetrapeptide are always negative over the temperature range under investigation. Comparing the results of thermodynamic experiments on model organic compounds it is apparent that the heat capacity change plays a central role in characterising the solvophobic interactions.[26a,28] The negative heat capacity changes can be attributed to the disordering of solvent molecules around the exposed solvophilic groups. The results given in Table 1 also suggest that the micelle formation of the tetrapeptide **1** is hindered by the increase in temperature, since the c.m.c. value of the peptide increases with increasing temperature. In an apolar medium like chloroform, the main interaction in the peptide aggregation is due to intermolecular hydrogen bond formation.[32] However, a large number of ΔG° values obtained from the literature[27b] (for amide–chloroform association) when plotted against *n* [number of −NHC(=O)− groups] led to a straight line with a slope of -5 ± 1 kJ mol^{-1} (due to the stabilization of amide groups of the peptide on interaction with chloroform molecules). In the present case, even though four amide groups are present, the $\Delta_m G^\circ$ value is maintained around −15 kJ mol^{-1} which is due to the larger contribution of negative $\Delta_m S^\circ$ to the aggregate formation.[24] On plotting $\Delta_m G^\circ$ *vs.* $T\Delta_m S^\circ$ (Fig. 8), the *y*-intercept indicates a $\Delta_m G^\circ$ value of −21.99 kJ mol^{-1} (which corresponds to $\Delta_m S^\circ = 0$), in agreement with the present interpretation.

The negative $\Delta_m S^\circ$ value may be due to a change in the number of chloroform molecules bound to the peptides on aggregation. However, on increasing the temperature both $\Delta_m H^\circ$ and $\Delta_m S^\circ$ decrease, indicating that more solvent molecules are accessible to the peptide, resulting in more 'structured' chloroform molecules. However, the negative entropic contribution seems to outweigh the negative $\Delta_m H^\circ$ contribution, which leads to aggregate destabilisation at higher temperatures. Thus, entropy and enthalpic effects compensate each other in such a way as to prevent the force of association from becoming too large. The apparent interpretation of this effect is that the release of solvent molecules from the peptide surface always leads to reduced solvent structure (which leads to $-\Delta_p C^\circ$).

The entropy–enthalpy compensation effect in the present system is well established by the plot of $\Delta_m H^\circ$ *vs.* $T\Delta_m S^\circ$ with the slope 0.8 (Fig. 9). Thus, it can be concluded that the micellisation of the peptide **1** in chloroform is stabilised by solvent peptide interactions rather than solvophobic interactions.

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