Fluorescent amino acids as reporter systems in peptido-cyclodextrin inclusion compounds

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The inclusion behaviour of mono-6-(*N*-acetyltyrosinyl)amino-6-deoxy-β-cyclodextrin and mono-6-(*N*-acetyltryptophanyl)amino-6-deoxy-β-cyclodextrin with regard to borneol, menthol and 5-methoxypsoralen have been investigated by fluorescence spectroscopy, circular dichroism and NMR spectroscopy. For mono-6-(*N*-acetyltryptophanyl)amino-6-deoxy-β-cyclodextrin, the weakly self-included indole residue is disincluded, and for 5-methoxypsoralen, fluorescence resonant energy transfer is observed. In contrast, for mono-6-(*N*-acetyltyrosinyl)amino-6-deoxy-β-cyclodextrin the self-inclusion is sufficiently strong that no disinclusion occurs. A binary complex is postulated to arise from hydrogen bonding between borneol and mono-6-(*N*-acetyltyrosinyl)amino-6-deoxy-β-cyclodextrin.

Introduction

Drug transport[1] and chiral separations[2] represent important applications of the cyclodextrins.[3] In both cases the grafting of amino acids or peptides onto the cyclodextrins is advantageous, either for vectorisation of drug transport to peptide receptors[4] or for the addition of a chiral auxillary which may enhance enantiomeric separation.[5] We have previously reported coupling D- and L-phenylalanine at the primary face of β-cyclodextrin **1**,[6,7] and in water there is formation of intramolecular inclusion complexes. Interestingly, when there is a Boc protecting group on the terminal amine, in the solid state for crystals grown from water, the phenyl group is not included and there is intermolecular complexation of the *tert*-butyl function.[8] This observation suggests that it should be possible to switch aromatic residues present on amino acids in and out of the cyclodextrin cavity in the presence of guest molecules.

The use of fluorescent reporter groups coupled to the cyclodextrins has been extensively developed by Ueno to yield systems in which the fluorescence signal is sensitive to inclusion processes, to external complexation, and to environmental changes around the cyclodextrins.[9] In the case of peptide-directed vectorisation of the cyclodextrins, the presence of tyrosine and tryptophan in the antenna may provide useful information on the environment around the vector and on the drug inclusion–disinclusion process. The quantum yields and maximum emission wavelengths are sensitive to the polarity of the environment around the fluorophore.[10] Our preliminary results suggested that for both the tyrosinylamido-β-CD and tryptophanylamido-β-CD compounds intramolecular inclusion occurred.[11] We were thus intrigued by a recent paper in which, based on NMR data for typtophanylamido-β-CD, it was proposed that the tryptophan amino acid function was situated outside the cavity even in the absence of a competitive guest molecule.[12]

In this paper we will show using fluorescence spectroscopy that, for mono-6-(*N*-acetyltyrosinyl)amino-6-deoxy-β-cyclodextrin **3** the aromatic group of the amino acid is too tightly bound within the cavity to be disincluded by borneol, a typical strongly bound terpene,[9,13] however for mono-6-(*N*-acetyltryptophanyl)amino-6-deoxy-β-cyclodextrin **4** the indole group is intramolecularly included but the binding is sufficiently weak that it might be disincluded by borneol or 5-methoxypsoralen.†[,14]

Experimental

Chemicals

Borneol, menthol, 5-methoxypsoralen (5-MOP) and ethanol (spectral grade) were obtained from Aldrich, water was triply distilled in glass. The modified cyclodextrins (CDs) were prepared as detailed below.

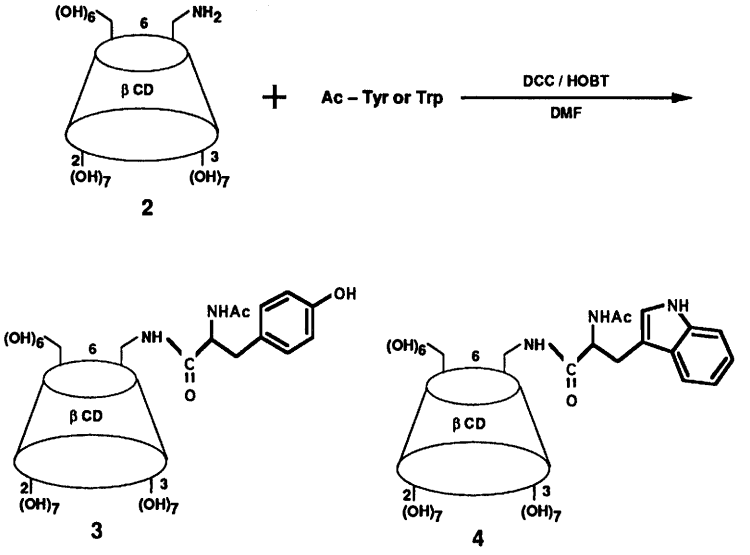
Synthesis

The mono-6-amino-6-deoxy-β-cyclodextrin **2** (1 g, 0.88 mmol) was coupled to the relevant *N*-acetyl amino acid (1.32 mmol) by the dicyclohexylcarbodiimine (DCC) (1 mmol)–hydroxybenzotriazole (HOBT) (1.76 mmol) procedure in DMF (10 ml) at room temperature. After two days, evaporation of the solvent and flash chromatography on a column of silica gel (with butanol–ethanol–H_2O, 5:4:3) gave the products **3** and **4** in good yields.

Mono-6-(*N*-acetyltyrosinyl)amino-6-deoxy-β-cyclodextrin 3. R_f 0.43, yield 82%; ^{1}H NMR [$(CD_3)_2SO$, 300 MHz]: δ_H 1.71 (s, 3 H, $COCH_3$), 2.8 (m, 2 H, Hβ Tyr), 3.3–3.8 (m, Hβ-CD), 4.60 (br d, OH-6 β-CD and Hα Tyr), 4.83 (d, 7 H, H-1 β-CD), 5.62–5.86 (br d, 14 OH β-CD), 6.62 (d, 2 H, $J_{H_A-H_B}$ 8.4 Hz, H_A Tyr), 7.02 (d, 2 H, H_B Tyr), 7.9 (br d, 1 H, NH β-CD), 8.08 (d, 1 H, $J_{NH-H\alpha}$ 7.8 Hz, NH Tyr), 9.2 (s, 1 H, OH Tyr); *m/z* (FAB) 1339 (M + 1).

Mono-6-(*N*-acetyltryptophanyl)amino-6-deoxy-β-cyclodextrin 4. R_f 0.41, yield 91%; ^{1}H NMR [$(CD_3)_2SO$, 300 MHz]: δ_H 1.72 (s, 3 H, $COCH_3$), 3.3–3.8 (m, H β-CD), 4.58 (br d, 8 H, OH-6 β-CD and CH_2 tryp), 4.81 (d, 7 H, H-1 β-CD), 5.64–5.85 (br d, 14 H, OH-2 and OH-3 β-CD), 6.96 (t, 1 H, $J_{H_B-H_A} = J_{H_B-H_C}$ 7 Hz, H_B Trp), 7.05 (t, 1 H, $J_{H_C-H_D}$ 7 Hz, H_C Trp), 7.09 (d, 1 H, *J* 2 Hz, H_E Trp), 7.3 (d, 1 H, $J_{H_A-H_B}$ 7 Hz, H_A Trp), 7.62 (d, 1 H, $J_{H_D-H_C}$ 7 Hz, H_D Trp), 7.96 (br d, 1 H, NH β-CD), 8.06 (d, 1 H, $J_{NH-H\alpha}$, NH amide), 10.75 (br d, 1 H, NH Trp); *m/z* (FAB) 1362 (M + 1).

† Psoralen = 7*H*-furo[3,2-*g*][1]benzopyran-7-one.



Scheme 1 Synthetic route to compounds **3** and **4**

Solutions

Cyclodextrin derivatives were prepared as stock solutions in water (10^{-3} mol l^{-1}). Borneol was dissolved in water to yield a stock solution of concentration (10^{-3} mol l^{-1}). 5-Methoxypsoralen was dissolved in ethanol to yield a stock solution (2×10^{-3} mol l^{-1}). Fluorescence and circular dichroism experiments were performed under identical conditions to permit direct comparison.

Spectroscopic measurements

Absorption spectra were measured using a Shimadzu 2100 UV–VIS spectrometer at ambient temperature (22 ± 2 °C) using a 10 mm quartz cell. Fluorescence spectra were measured on a Perkin-Elmer LS-50B spectrometer using a 10 mm quartz cell. The emission and excitation widths were set at 5 and 3 nm, the excitation wavelength was 280 nm.

^{1}H NMR spectra were obtained in $(CD_3)_2SO$ and D_2O on a Bruker 300 MHz and Varian 500 MHz spectrometer respectively.

Circular dichroism spectra were recorded on a Jobin Yvon CD6 spectrometer.‡

Results and discussion

The two fluorescent β-cyclodextrin derivatives mono-6-(*N*-acetyltyrosinyl)amino-6-deoxy-β-cyclodextrin **3** and mono-6-(*N*-acetyltryptophanyl)amino-6-deoxy-β-cyclodextrin **4** were synthesised from mono-6-amino-6-deoxy-β-cyclodextrin **2**[6] and the corresponding *N*-acetyl amino acid using a standard HOBT–DCC coupling procedure (Scheme 1).[15]

The choice of the *N*-acetyl derivatives was based on the need to replicate as closely as possible the chemical environment that would be obtained from coupling a peptide or glycopeptide to the cyclodextrin carrier. It has previously been shown for the *N*-acetylphenyl alaninyl-β-cyclodextrin that there is little difference in the self-inclusion behaviour as compared to the simple phenylalaninyl-β-CD derivative.[6] Molecular modelling studies suggest that the the amine or amide function will be directed out of the cavity and should not participate in the complex hydrogen bonding and hydrophobic interactions involved in the self-inclusion process.[16]

After isolation of the desired products, they were suspended in acetone and refluxed for 8 h. This procedure was repeated three times to eliminate any residual HOBT that might be present as an inclusion complex. The use of fluorescence spectroscopy, which is highly sensitive with regard to the detection of low quantities of impurity, has shown that such treatment is necessary.[11] Other physical techniques, such as NMR, are unfortunately probably incapable of detecting impurities present at less than 5–10%.

The fluorescence emission spectra of **3** and **4** are given in Fig. 1(*a*) (**3**) and 1(*b*) (**4**). The maximum wavelength of fluorescence emission is 300 nm for **3** and 349.5 nm for **4**; both values show a blue shift in wavelength with regard to the free amino acids (305.5 nm, 362 nm). This blue shift is diagnostic of the fluorophore being located in a more hydrophobic environment, which we believe to be the cyclodextrin cavity in both cases. The values are insensitive to variation in the concentration of **3** or **4**, which leads us to propose that self-inclusion occurs in both cases. The quantum yields (φ_F) observed (0.087 for **3** and 0.21 for **4**) are much lower than those observed for the free ligands (0.14 and 0.33, respectively). In general inclusion of fluorophores in the hydrophobic cyclodextrin cavity gives rise to an increase in the emission intensity, however both increases and decreases are often observed in proteins and we believe here the conformational changes imposed by self-inclusion give rise to this decrease.

Our findings are in accord with the NMR studies of Djediaini *et al.*[12] for the tyrosine derivative **3** but are in apparent contradiction for the tryptophan derivative **4**. It should be noted that the one dimensional ^{1}H NMR spectrum of **4** shows the peak splitting typically arising from symmetry breakdown due to self-inclusion. If the self-inclusion complex has a low association constant, there will a large number of conformational isomers as the tryptophan sweeps out a volume between the extremes of self-inclusion and total disinclusion and that no single isomer has a sufficient lifetime to contribute significantly to NOE effects, hence no correlation peaks will be observed.[12]

The difference in the association constants for self-inclusion of the amino acid moiety for **3** and **4** can clearly and easily be demonstrated using fluorescence spectroscopy in the presence of guest molecules. The effects on the quantum yields and fluorescence emission wavelengths will also provide definitive information on the self-inclusion properties of **4**.

In Fig. 2 is presented the variation in fluorescence intensity for **3** and **4** in the presence of borneol, as a function of borneol concentration. Borneol was chosen as a strongly including guest

‡ NMR and CD spectra are available as supplementary data (SUPPL. NO. 57149) from the British Library. For details of the Supplementary Publications Scheme, see Instructions for Authors, *J. Chem. Soc., Perkin Trans. 2*, 1996, Issue 1.

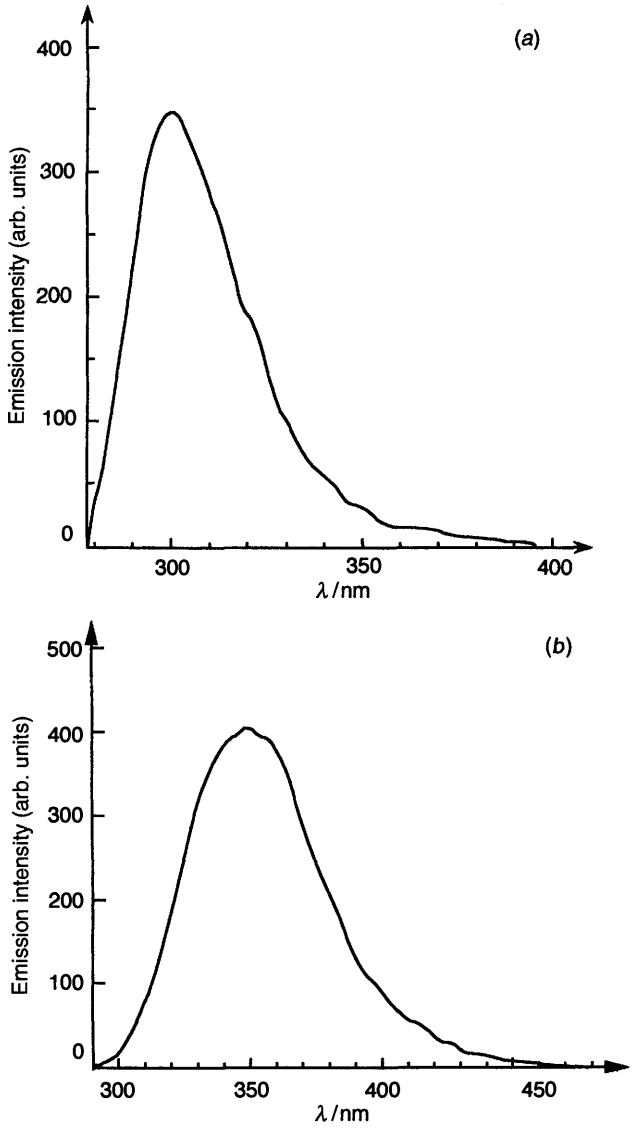


Fig. 1 Fluorescence emission spectra of (*a*) compound **3** and (*b*) compound **4**

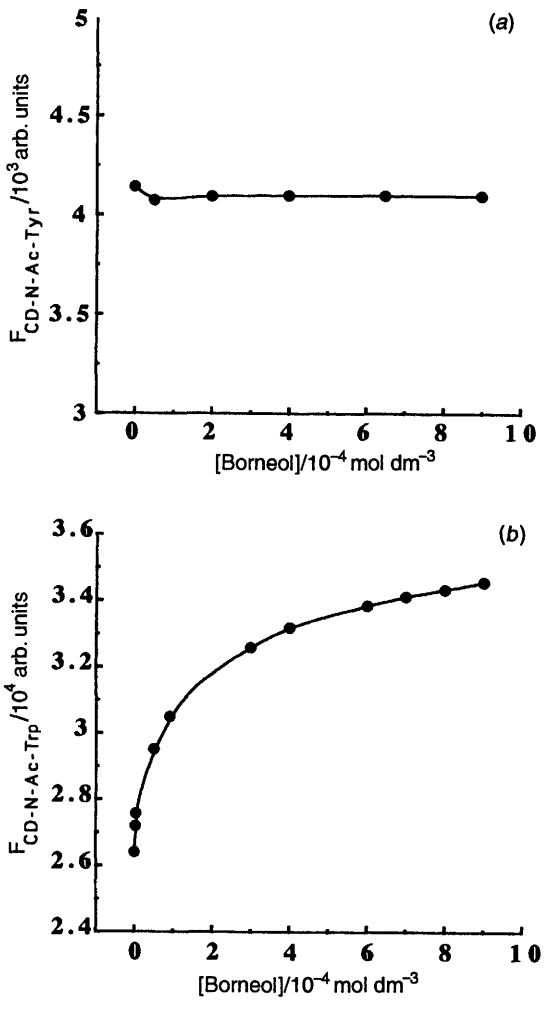


Fig. 2 Fluorescence intensity variation of **3** and **4** as a function of borneol concentration

as shown by guest competition experiments[13] and also in competition experiments with a rim-appended fluorophore.[9] Borneol is a non-fluorophore and thus no effects due to fluorescence resonance energy transfer[17] are expected.

For complex **4** the emission intensity rises in an asymptopic manner up to the saturation concentration of borneol at 9×10^{-4} mol l^{-1}; this shows a disinclusion process for the self-included tryptophan in which it is replaced by borneol.

This is further confirmed by the red-shift in the emission maximum length which changes from 349.5 to 358 nm, and shown in Fig. 3 are the fluorescence spectra of **4** at the extreme limits of Borneol concentration used.

In contrast, for **3** no variation in either the fluorescence intensity or the emission maximum wavelength is observed, hence the self-inclusion complex is apparently too strongly bound for borneol to replace the included tyrosine moiety.

Again, the fluorescence measurements are in agreement with the NMR results for the tyrosine derivative **3**, but fluorescence spectroscopy clearly demonstrates that for the tryptophan, there must exist a weak dynamic self-inclusion.

The second guest molecule is 5-MOP, which shows strong enhancement of its fluorescence spectra when included in the β-cyclodextrin cavity.[14] More importantly, the absorption spectrum of 5-MOP strongly overlaps with the emission spectrum of tryptophan, as shown in Fig. 4.

From this we can expect good fluorescence resonant energy

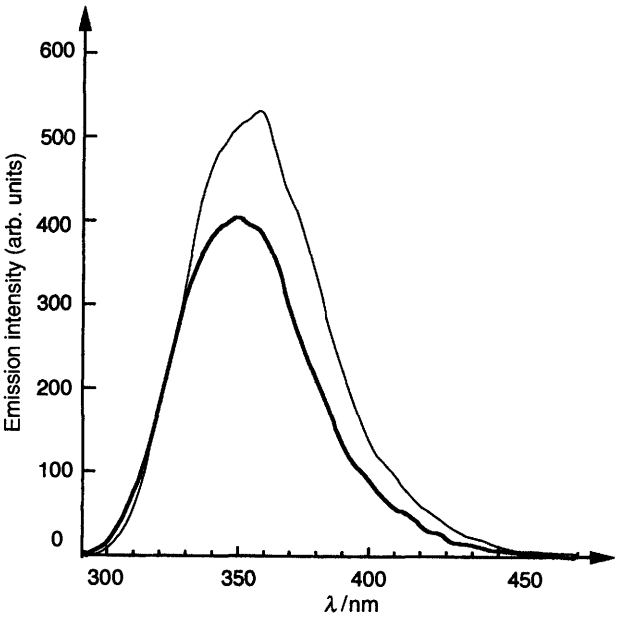


Fig. 3 Fluorescence spectra of **4** at zero (—) and maximum (9×10^{-4} mol l^{-1}) concentration of borneol (—)

transfer (FRET, or the Förster effect)[17] to occur if the 5-MOP is included in the cavity. Then energy will be transferred from the excited tryptophan residue to the included 5-MOP and in consequence the emission intensity will be quenched.

In Fig. 5 the emission intensity of the tryptophan residue of **4**

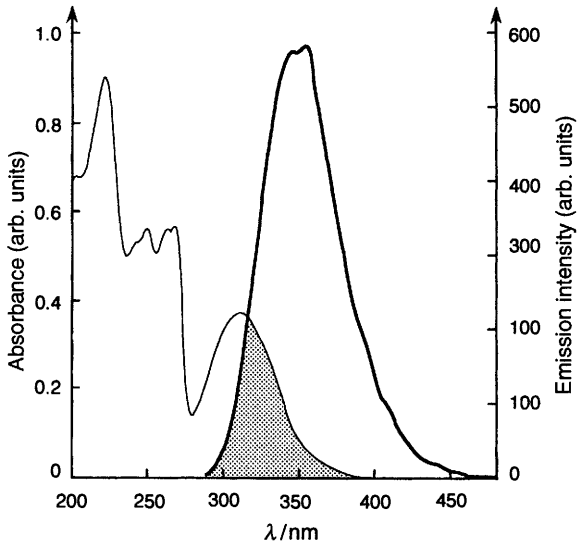


Fig. 4 Emission fluorescence spectra of **4** (—) and absorption of 5-MOP (—) between 200 and 500 nm (the spectral overlap region is shaded)

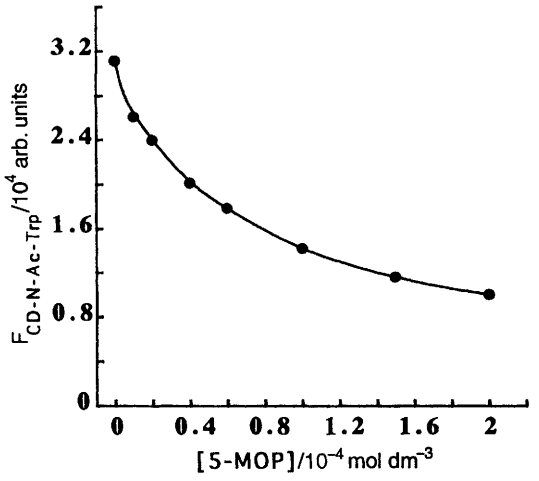
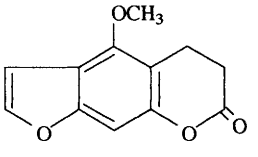


Fig. 5 Fluorescence emission intensity of **4** as a function of 5-MOP concentration

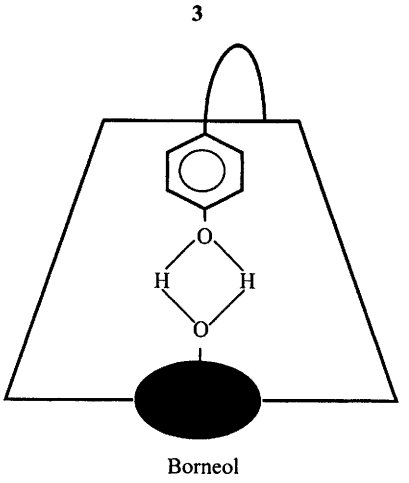


5-MOP

is shown as a function of 5-MOP. This experiment was carried out using a H_2O–ethanol (9:1) solution to solubilise the 5-MOP. In a blank experiment ethanol was shown to augment slightly the fluorescence intensity, which could be expected from formation of a tertiary complex as previously shown by Warner for pyrene–cyclodextrin–alcohol systems.[18] It is evident that a strong FRET effect takes place, showing that the two fluorophores are relatively close spatially. The exact distance is not calculated, as the R_0 value may not be obtained. This would require the absolute magnitude of the intensity for the disincluded tryptophan fluorophore to be determined, which, given that we are dealing with equilibria, is not obtainable.

As for borneol, 5-MOP has no effect on the fluorescence properties of **3**, showing again the extremely strong self-inclusion of the tyrosine residues.

The interaction between guest species and **3** or **4** has been further studied by using circular dichroism.[10] For **4**, the band at 225 nm arising from the tryptophan residue was observed; in



Borneol

Fig. 6 Postulated hydrogen-bonded binary complex between **3** and borneol

the case of **4** alone a positive band was observed, while in the presence of borneol the intensity of this band was strongly diminished and a second negative band at 200 nm appears. This is consistent with the tryptophan residue being displaced from the cavity. Interestingly, for menthol, a terpene which Ueno[9] and ourselves[13] have observed to be less strongly bound than borneol, a much smaller effect is observed at equivalent terpene concentrations.

In the case of **3** a positive band at 230 nm is observed, for which a slight decrease in intensity is observed in the presence of borneol. However, in the presence of borneol a strong positive band appears at 205 nm. We postulate that this may arise from the presence of a binary complex **3**:borneol, formed by hydrogen bonding between the phenolic hydroxy group of tyrosine and the borneol hydroxy group.

In order to probe further the self-inclusion properties, preliminary ^1H NMR studies of **3** and **4** have been undertaken in D_2O. In the case of **4** in 1D experiments, for the complex alone the signals arising from the anomeric protons are well separated, appearing as six peaks at δ 5.08, 5.03, 5.00, 4.96, 4.93 and 4.88. In the presence of an excess of borneol the spectral pattern is simplified with only five anomeric resonances observed (δ 5.08, 5.03, 5.00, 4.96, 4.93). Spectral changes are also observed in the aromatic region and the complex group of resonances from the other cyclodextrin protons are much simplified. These results are in accord with exclusion of the tryptophan residue from the cyclodextrin cavity.

For **3** alone, four anomeric resonances are observed, but no changes are observed in the presence of an excess of borneol. However, in the aromatic region the peak at δ 7.00 arising from the protons *ortho* to the phenolic hydroxy group are displaced by 0.1 ppm and the signal is broadened, while the other aromatic protons remain unchanged. This is consistent with formation of the binary complex postulated from circular dichroism experiments. For both **3** alone and in the presence of borneol, the region between δ 3 and 4 arising from the other cyclodextrin protons remains complex, in agreement with the tyrosine residue being retained within the cavity. The results on the free peptido-β-cyclodextrin systems are in accord with those observed by Djedaini for the non-*N*-acylated derivatives.[12]

Guest species are thus able to displace the weakly self-included tryptophan from the cavity, however the more strongly bound tyrosine is not displaced. In the NMR studies previously reported sodium anthraquinone-2-sulfonate cannot hydrogen bond to the tyrosine hydroxy group, so the postulated binary complex observed for borneol will not occur.

Conclusions

We have shown that fluorescent reporter moieties attached to the upper rim of β-CD can be used to detect inclusion–disinclusion effects, and that use of both reporter and guest fluorophores gives rise to efficient fluorescence resonance energy transfer. We are currently investigating the intriguing possibility of hydrogen-bonded binary complexes involving the self-included tyrosine and the results will be reported in due course. It should be noted that abiotic fluorophores as developed by Ueno[9] will be more ideally suited to the use of such systems to detect transport of bio-active molecules into living cells.

Acknowledgements

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