Journal of Pharmaceutical & Biomedical Analysis
Vol. 10, No. 1, pp. 23–30, 1992
Printed in Great Britain

0731–7085/92 $5.00 + 0.00

New fluorescent derivatives of cyclosporin for use in immunoassays

M.T. FRENCH,* J.N. MILLER,* N.J. SEARE,*† D.R. LACHNO‡ and M.H. YACOUB‡

* *Department of Chemistry, Loughborough University, Ashby Road, Loughborough, Leicestershire LE11 3TU, UK*
‡ *Thoracic and Cardiac Surgical Unit, Harefield Hospital, Middlesex UB9 6JH, UK*

Abstract: The synthesis of new fluorescent derivatives of cyclosporin is described and their affinity with the specific Sandoz monoclonal antibody investigated. Synthesis was carried out using cyclosporin-C-hemisuccinate as the starting material with monodansylcadaverine, 4-bromomethyl-7-methoxycoumarin, and 4-bromomethyl-6,7-dimethoxycoumarin as labels. After extraction the derivatives were purified by HPLC and their binding affinity with the monoclonal antibody evaluated by the Incstar Cyclo-Trac SP radioimmunoassay. All three derivatives showed good binding and it is suggested that they may be of use in an immunoassay for measuring cyclosporin.

Keywords: *Fluorescence immunoassay; cyclosporin; fluorescent derivatives; monoclonal antibodies.*

Introduction

Cyclosporin A (Fig. 1) is an extremely powerful immunosuppressive drug whose uses include the prevention of organ rejection in transplantation [1] and the treatment of some autoimmune diseases [2] and parasitic infections [3]. In transplantation therapy it acts by selectively inhibiting the rejection process, allowing patients to live a near normal life. However the therapeutic range is narrow and ill defined, varies between individuals, and is dependent on a number of factors including the assay method, the sample matrix and the post-transplant time [4]. Routine monitoring is therefore essential if therapy is to be optimized.

The currently available assay techniques include high-performance liquid chromatography (HPLC), radioimmunoassay (RIA with both monoclonal and polyclonal antibodies) and fluorescence polarization immunoassay (FPIA) [5–7]. These provide conflicting results and correlation between them is difficult since some methods determine only the parent compound whereas others measure both parent compound and varying amounts of metabolites, giving inflated assay results. This is problematic because the immunosuppressive activity and toxicity of the metabolites is still not fully understood [7]; parent compound: metabolite ratios are patient-specific and greatly influenced by the organ transplanted.

The introduction of monoclonal antibodies has promoted immunoassay as the method of choice in most cases rather than the more time-consuming and technically complex HPLC techniques [8]. However the routine immunoassays available have many shortcomings. RIA analysis gives concern in the areas of environmental and health protection as well as having a requirement for highly trained technical staff carrying out lengthy and numerous handling steps. FPIA requires costly equipment. Both methods are suitable for large laboratories analysing many samples but the methods are inconvenient for use on patients who have returned to the community.

The present work involves the synthesis and evaluation for use in immunoassays of fluorescent derivatives of cyclosporin using new fluorophores in an attempt to overcome some of the problems traditionally associated with fluorescein, such as small Stokes shift.

Experimental

Chemicals and reagents

All cyclosporin congeners were kind gifts from Sandoz (Basle, Switzerland); monodansylcadaverine (MDC), *N,N'*-dicyclohexylcarbodiimide (DCCI) and 4-bromomethyl-7-

† Author to whom correspondence should be addressed.

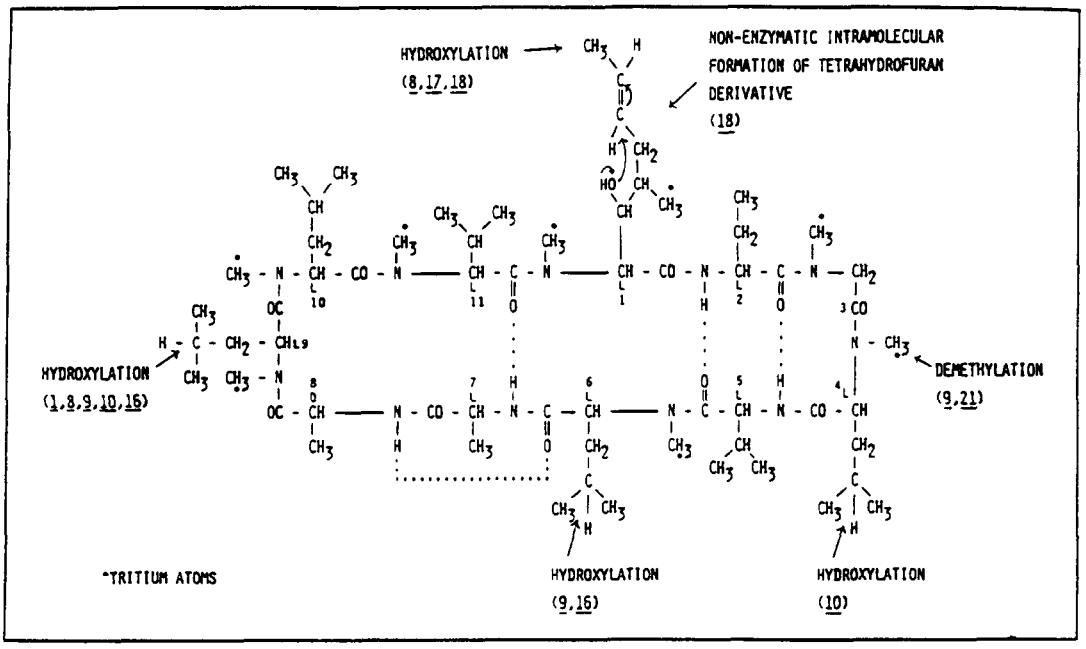

Figure 1
Structure of cyclosporin A showing sites of metabolism.

methoxycoumarin (BMMC) were purchased from Sigma (Poole, Dorset, UK). Other reagents used were 18-crown-6 (Aldrich, Gillingham, Dorset, UK), anhydrous potassium carbonate (BDH, Poole, Dorset, UK) and 4-bromomethyl-6,7-dimethoxycoumarin (BMDC) (Kodak, Liverpool, UK). All organic solvents were HPLC grade and purchased either from Fisons chemicals (Loughborough, Leicestershire, UK) or Rathburn chemicals (Walkerburn, Peebleshire, UK).

Apparatus

Chromatography was performed as described by Lachno *et al.* [9] except that a Merck–Hitachi F1000 fluorescence detector was placed in series with and after the UV detector while the AASP (advanced automated sample processor) was used only to inject the sample via the pneumatically operated valve (Valco Instruments) and at the same time initiate data collection for each detector. The mobile phase comprised ethanol–hexane (15:85, v/v), the flow rate was set at 1.0 ml min^{-1}, and 25-μl samples were injected. The fluorescence detector excitation (Ex) and emission (Em) wavelengths were set for each derivative as follows: MDC — Ex 340 nm/Em 490 nm; BMMC — Ex 355 nm/Em 410 nm; and BMDC — Ex 380 nm/Em 430 nm. All fluorescence spectra were recorded uncorrected at room temperature using a Perkin–Elmer LS50 fluorescence spectrometer with data manipulation and processing carried out using a fluorescence data management system (Perkin–Elmer Beaconsfield, Buckinghamshire, UK) operating on an Epson AX3 personal computer. Gamma counting was carried out using an LKB 1282 Compugamma instrument with data handling and curve fitting using the LKB RIACALC software. All IR spectra were taken in chloroform on a Perkin–Elmer 1600 series FTIR spectrometer interfaced to an Epson FX 850 printer. TLC was carried out using Kieselgel 60 F_{254} aluminium backed sheets of 0.2-mm thickness. Preparative plates of 1-mm thickness were prepared using Kieselgel 60 GF_{254}. Both types of plate were obtained from Merck (Darmstadt, Germany).

Procedures

Synthesis of fluorescent derivatives.

Synthesis using monodansylcadaverine (MDC). A 38.1 mg volume of MDC and 10.0 mg of dicyclohexylcarbodiimide were dissolved in 40 ml of dried CH_2Cl_2 in a 100-ml round-bottomed flask. To this solution was added dropwise with stirring 51.8 mg of cyclosporin-C-hemisuccinate dissolved in 20 ml of dried CH_2Cl_2; the mixture was left in the dark for 24 h. The solvent was then removed by

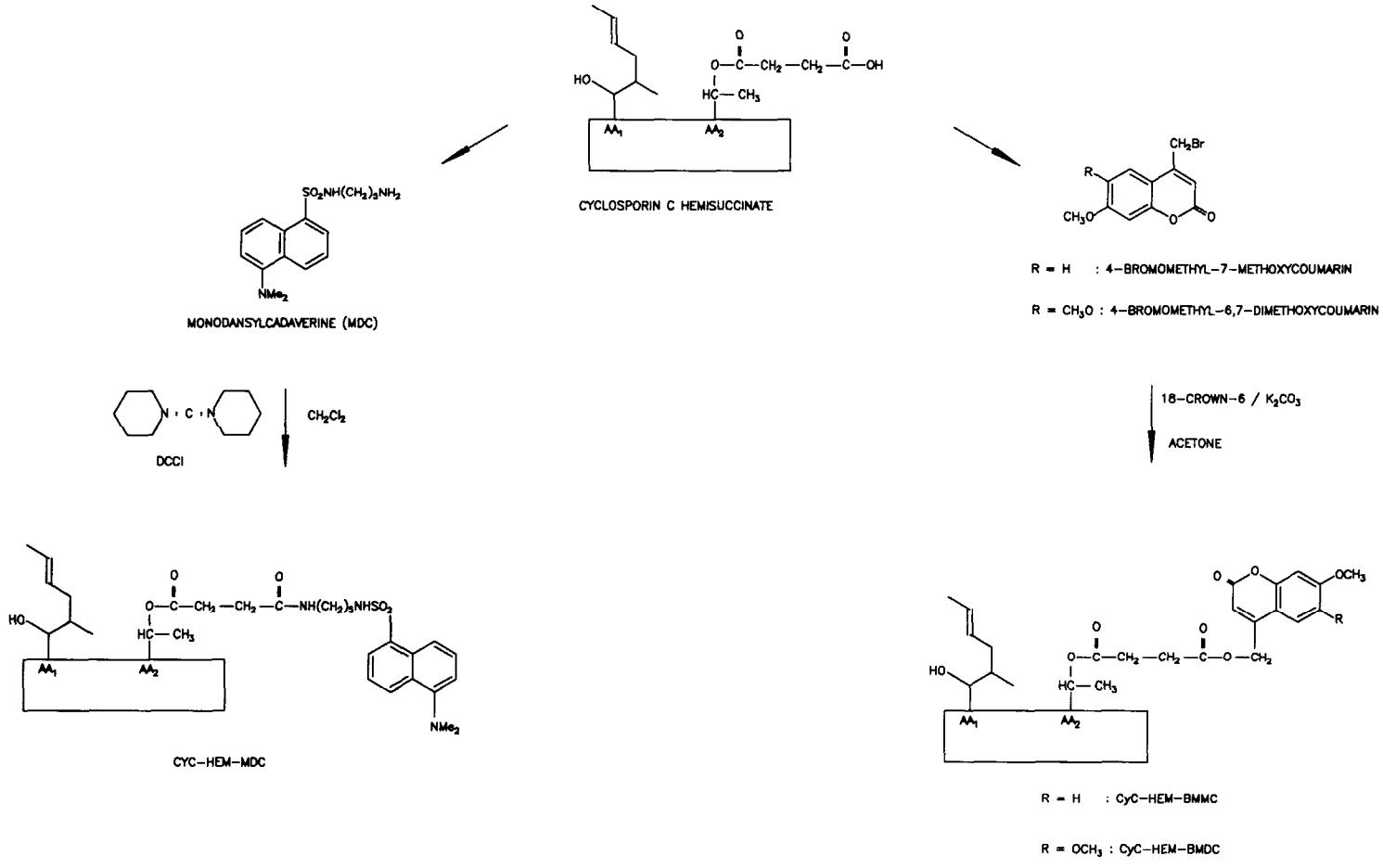

Figure 2
Reaction scheme for the synthesis of fluorescent derivatives of cyclosporin-C-hemisuccinate.

evaporation and the products of interest were isolated by TLC.

Synthesis using 4-bromomethyl-6,7-dimethoxycoumarin (BMDC) or 4-bromomethyl-7-methoxycoumarin (BMMC). A 24.1 mg volume of cyclosporin-C-hemisuccinate, 3.0 mg of 18-crown-6 and 13.5 mg of anhydrous potassium carbonate were added to a round-bottomed flask. The constituents were then dissolved in 20 ml of a solution of BMDC or BMMC in acetone (0.84 mg ml^{-1}) and the mixture refluxed for 1 h. The solvent was then removed by evaporation and the product of interest isolated by TLC.

Isolation and extraction of derivatives. Each reaction mixture was examined by TLC using a solvent system of $CHCl_3$–MeOH (96:4, v/v) with UV visualization of the fluorescent derivatives at 366 nm. The same solvent system was then used with preparative TLC to isolate the products, which were washed from the silica with methanol.

HPLC analysis and isolation of fluorescent derivatives. For each derivative 25 μl of crude extract was injected on to the column and monitored by UV and fluorescence detection. Fluorescent fractions were collected and the mobile phase evaporated off under reduced pressure after which the derivative was reconstituted into 10 ml of ethanol and 50% serial dilutions made. Duplicate 50-μl aliquots were then submitted for RIA analysis.

RIA analysis. This was carried out using the Incstar Cyclo-Trac SP ^{125}I RIA kit containing monoclonal antibodies specific for the parent compound, cyclosporin A. The assay was carried out according to the manufacturer's instructions except that the samples were assayed without pre-dilution.

Results and Discussion

All metabolites of cyclosporin A so far identified differ from the parent compound by changes to one or more residues at positions 1, 4, 6 and 9 only [10]. These residues are contained within the epitope recognized by the Sandoz 'specific' monoclonal antibody [11]. Therefore any fluorescent derivative of cyclosporin must not have changes to its structure or conformation at these positions if it is to be bound by the antibody.

Cyclosporin C differs from cyclosporin A in that it has a threonine residue at position 2. The —OH group thus available provides a reactive centre through which conjugation to suitable fluorophores is possible. As this mirrors the approach used to raise the monoclonal antibody to cyclosporin A [11] any fluorescent group at position 2 should not be within the recognition epitope and the antibody should bind the derivative in the same way as the parent compound.

Cyclosporin-C-hemisuccinate was used as the starting material since it was reasoned that the hemisuccinate bridge would act as a spacer between the cyclosporin molecule and the fluorophore and so help to reduce quenching effects due to steric hindrance.

Three derivatives have been prepared using: monodansylcadaverine (MDC); 4-bromomethyl-7-methoxycoumarin (BMMC); and 4-bromomethyl-6,7-dimethoxycoumarin (BMDC). MDC did not give a clean product when examined by TLC. Two fluorescent spots were seen on the chromatogram and were isolated and examined by HPLC. The faster migrating spot was found to consist of two distinct fluorescent compounds whereas the other spot contained only one compound. It is suggested that the former spot was a single compound that degraded on the column to give two fractions and so should be discarded. The latter spot was used for all subsequent work with this derivative.

Two more derivatives were synthesized using BMMC and BMDC. The reaction mixtures were examined by TLC and each showed only a single fluorescent product; this was confirmed by HPLC (Fig. 3). The fluorescent product from each reaction mixture was isolated by preparative TLC, weighed, reconstituted into a known volume and its spectral properties examined in methanol. For each derivative the excitation (Ex) and emission (Em) maxima were found to be: CyC–hem–MDC, Ex 336 nm/Em 510 nm; CyC–hem–BMMC, Ex 355 nm/Em 409 nm; CyC–hem–BMDC, Ex 383 nm/Em 439 nm.

These values show that the coumarin derivatives, BMMC and BMDC have Stokes shifts (the difference between their excitation and emission maxima) of only about 50 nm whereas the MDC derivative has a shift of 174 nm. A large Stokes shift is important since

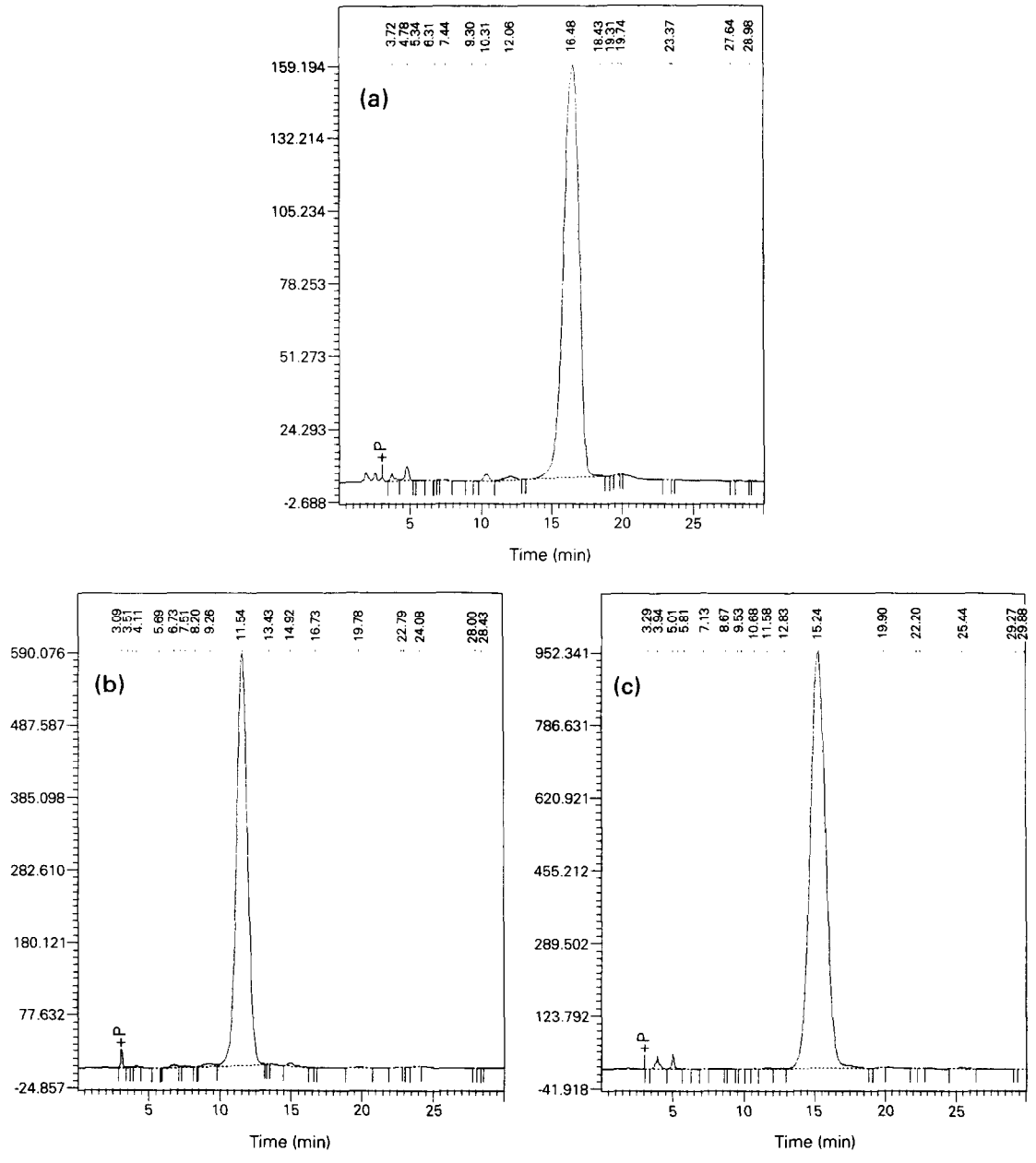

Figure 3
HPLC chromatograms with fluoroscence detection of (a) CyC–hem–MDC, (b) CyC–hem–BMMC and (c) CyC–hem–BMDC. Column: 5-μm CPS Hypersil. Column temperature: 53°C. Mobile phase: ethanol–hexane (15:85, v/v). Flow rate: 1.0 ml min^{-1}.

interference due to scattered light increases if the excitation and fluorescence wavelengths are too similar, thus limiting the sensitivity of the assay. The derivatives were also examined in methanol–phosphate buffered saline (pH 7.4) (PBS) (5:95, v/v); the excitation and emission spectra of CyC–hem–BMDC are shown in Fig. 4.

The infra-red spectra of each derivative in $CHCl_3$ showed characteristic cyclosporin peaks at 1635 cm^{-1} (amide carbonyl) and at 970 cm^{-1} (C–H deformation on double bond in C9 amino acid). The fluorophore contributed a band centred at 3020 cm^{-1} (aromatic C–H stretch). A typical infra-red spectrum for CyC–hem–BMMC is shown in Fig. 5.

Once the derivatives had been synthesized and isolated they were purified by HPLC ready for analysis by monoclonal RIA. The chromatogram for each derivative showed a single

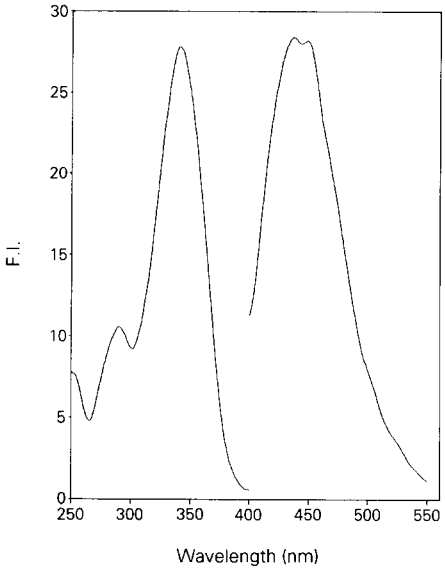

Figure 4
Excitation and emission spectra. Cyc-hem-BMDC in MeOH-PBS (pH 7.4) (5:95, v/v).

fluorescent product with good symmetrical peak shape indicative of peak purity. The mobile phase was monitored by a UV and fluorescence detector and the fraction containing the fluorescent derivative was collected. The solvent was removed by evaporation and the product reconstituted in 10 ml of ethanol.

Serial dilutions (50%) were made for each derivative and duplicate 50-μl samples of each were analysed by RIA. The results are summarized in Table 1.

The calibration curve for the RIA is at its most reliable between 100 and 500 ng ml^{-1} (private communication — Harefield Hos-

Table 1
RIA results for the serial dilution of the cyclosporin derivatives

Sample	Dilution factor	Concentration (ng ml^{-1})
MDC 1	0	458
MDC 2	1:1	111
MDC 3	1:2	43
MDC 4	1:4	43
MDC 5	1:8	30
MDC 6	1:16	16
MDC 7	1:32	10
BMMC 1	0	3318
BMMC 2	1:1	2783
BMMC 3	1:2	1162
BMMC 4	1:4	487
BMMC 5	1:8	232
BMMC 6	1:16	97
BMMC 7	1:32	58
BMDC 1	0	2586
BMDC 2	1:1	2488
BMDC 3	1:2	815
BMDC 4	1:4	410
BMDC 5	1:8	199
BMDC 6	1:16	88
BMDC 7	1:32	49

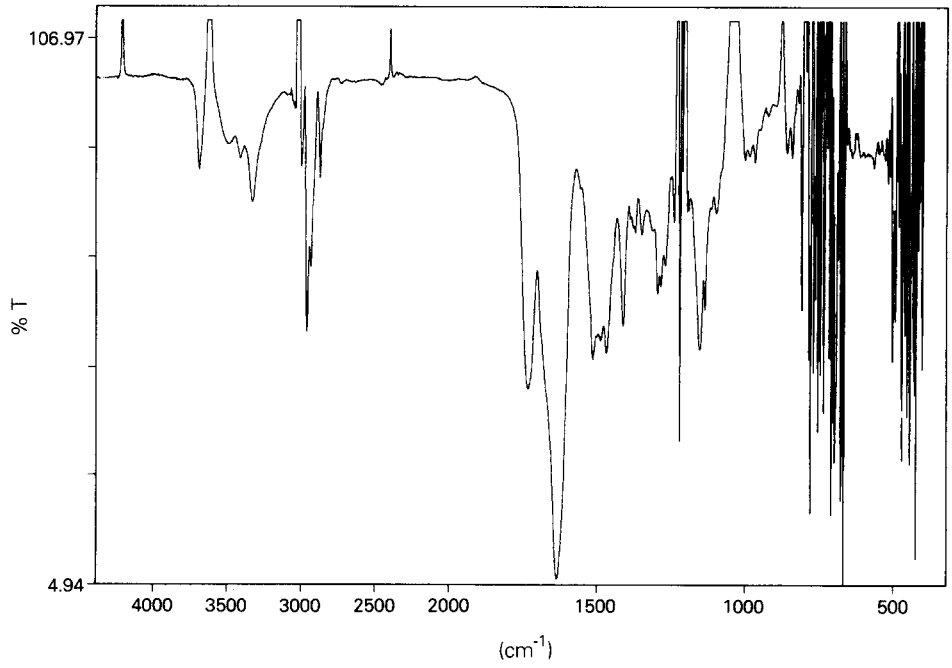

Figure 5
IR spectrum of CyC-hem-BMMC in $CHCl_3$.

pital). The results show that all three derivatives exhibit binding with the antibody and that for the BMMC and BMDC derivatives the serial dilutions are represented in the results within the calibration range. The MDC derivative shows poor results although for the most part they are outside the optimum calibration range. The low values may be due to low binding affinity with the antibody; in further studies the concentration of each stock solution was calculated using the RIA results and compared with those obtained by dry mass calculations (Table 2).

From these results it can be seen that a much smaller percentage of the crude CyC–hem–MDC product is detected by RIA than that detected with the coumarin derivatives supporting the suggestion of lower affinity of CyC–hem–MDC with the monoclonal antibody.

Using concentration values determined by RIA some estimation of the overall yield of each synthesis was possible. For the MDC derivative the reaction gave less than 1% yield, reflecting the generation of multiple products. For the coumarin derivatives the overall yield was approximately 33 and 46% for the BMMC and BMDC products, respectively. These two yields might be further enhanced by changing the reaction conditions although in determining the yields it has been assumed that the monoclonal antibody has the same affinity for all derivatives, an assumption that may not be valid.

During the course of any fluorescence measurements the sample is exposed to an intense light source and in some cases the level of incident light is sufficient to decompose the sample. Therefore investigations were carried out to compare the photostability of the derivatives with that of a solid fluorescent standard (tetraphenyl butadiene in polymer matrix), and that of fluorescein, a very widely used and accepted fluorophore. Intensity measurements were taken with a methanolic sample continuously illuminated for 10 min and the results plotted as a percentage of the original intensity (Fig. 6).

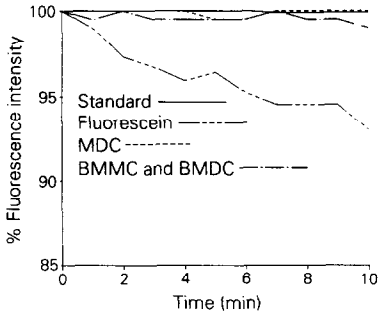

Figure 6
Comparison of the photostability of the cyclosporin derivatives with fluorescein and a solid fluorescent standard.

These results show that the photodecomposition of all the derivatives is less than that of fluorescein; the MDC derivative clearly shows the best photostability. It would seem from this evidence that in the use of these derivatives in immunoassays, photodecomposition should not be a problem while readings are being taken. Further studies on the longer term stability of the derivatives in subdued light are in progress.

Conclusions

The aim of the work was to produce fluorescent labelled cyclosporin derivatives for use in a fluoroimmunoassay. This has been achieved with the synthesis of three photostable derivatives of cyclosporin-C-hemisuccinate, all showing some degree of compatibility with the monoclonal antibody. Of the three, the coumarin derivatives show the most promise since the reaction yield and antibody binding of CyC–hem–MDC is low.

The immunological compatibility between the monoclonal antibody and the derivatives suggests that each synthesis has proceeded as expected since the antibody would be expected to discriminate against derivatives labelled at any other point on the cyclosporin molecule,

Table 2
Comparison of the product yield as determined by dry mass and RIA measurements

Derivative	'Dry mass' (x) (mg)	'RIA mass' (y) (mg)	$\frac{y}{x} \times 100$ (%)
Cyc–hem–MDC	1.70	0.18	10.6
CyC–hem–BMMC	10.00	7.80	78.0
CyC–hem–BMDC	16.70	10.95	65.6

while the HPLC analysis suggests that each derivative is a single compound.

References

[1] T. Beveridge, *Prog. in Allergy* **38**, 269–292 (1986).
[2] M. Schmitz-Schumann, *Prog. in Allergy* **38**, 436–446 (1986).
[3] J.F. Borel, *Transplant Proc.* **15** (Suppl. 1), 2219–2229 (1983).
[4] R.J. Ptachcinski, *J. Clin. Pharmacol.* **26**, 358–366 (1986).
[5] M.C. Haven, *Clin. Chem.* **35**, 564–568 (1989).
[6] A. Sanghvi, *Clin. Chem.* **34**, 1904–1906 (1988).
[7] Report of the task force on cyclosporin monitoring, *Clin. Chem.* **33**, 1269–1288 (1987).
[8] V. Quesniaux, *Clin. Chem.* **33**, 32–37 (1987).
[9] D.R. Lachno, N. Patel, M.L. Rose and M.H. Yacoub, *J. Chrom.* **525**, 123–132 (1990).
[10] M. Lemaire, *Prog. in Allergy* **38**, 93–107 (1986).
[11] V. Quesniaux, *Molec. Immun.* **24**, 1159–1168 (1987).

[Received for review 21 November 1990; revised manuscript received 18 July 1991]