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# A Practical Method for the Synthesis of a Cyclosporine−Fluorescein Conjugate

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## Abstract:

**A straightforward synthesis of a cyclosporine−fluorescein conjugate through the MeBmt hydroxyl group was developed. Reaction of cyclosporine A in phosgene/toluene solution with added triethylamine, followed by subsequent reaction with 4′-aminomethylfluorescein, produces good yields of the cyclosporine−fluorescein conjugate.**

## Introduction

Since it was isolated in 1967 from the fungus *Tolypocladium iinflatum*,[1] cyclosporine A (CsA, Figure 1) has been shown to be a powerful immunosuppressant which prevents rejection of heart, kidney, pancreas, lung, intestine, liver, and bone marrow transplants in humans.[2] Due to its potential to cause severe nephrotoxicity, hypertension, neurotoxicity, and seizures,[3] cyclosporine A has been the subject of intense clinical study aimed at determining the optimal method for drug analysis in both serum and, more commonly, in whole blood.[4] Therapeutic drug monitoring is required to identify patients with poor drug absorption, unusual metabolism or clearance, changes in bioavailability and saturation, and to adjust post-transplant dosage to prevent insufficient immunosuppression or drug-induced toxicity.[5]

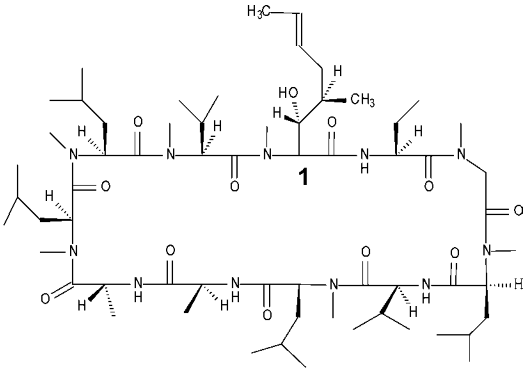

*Figure 1.*

Immunoassays using radiolabeled[6,7] or fluorescently labeled[8−10] cyclosporine are preferable as analytical methods, due to their rapid assay time and technical ease. These assays have been particularly useful in discriminating between CsA and its generally inactive metabolites[11] due to the employment of selective monoclonal antibodies that show specificity, reproducibility, and sensitivity.[11,12] In the case of covalent labeling reactions, synthetic processes for CsA conjugates are lengthy[13] or low yielding,[14] particularly for coupling via the MeBmt[15] residue due to steric factors caused by CsA's three-dimensional conformation.[13,16] We report here a straightforward, scaleable process that provides reproducible yields of a CsA−fluorescein conjugate linked through the hydroxy group of the MeBmt.

## Results and Discussion

At the onset of this work, the chloroformylation was conducted in the manufacturing area by reacting CsA with a 20% solution (w/w, $\sim$1.9 M) of phosgene in toluene (Fluka) for 18 h (ambient), producing low yields (variable $\sim$7%) of chloroformate as determined by subsequent reaction (see entry 1, Table 1).[17]

The coupling reaction of the chloroformate with 4′-aminomethylfluorescein (Scheme 1) thus produced low yields of the labeled conjugate. Until process optimization was complete, we standardized on 100 mg of CsA and a 30-mL reaction volume (needed to dissolve CsA, $\sim$693 equiv of phosgene) to minimize process variation. Due to the corrosiveness of phosgene (NOTE: phosgene is very corrosive,

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(15) MeBmt is an abbreviation for the amino acid residue of (4*R*)-4-[(*E*)-2-butenyl]-4,*N*-dimethyl-L-threonine, usually numbered in cyclosporine as position 1.

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***Scheme 1***

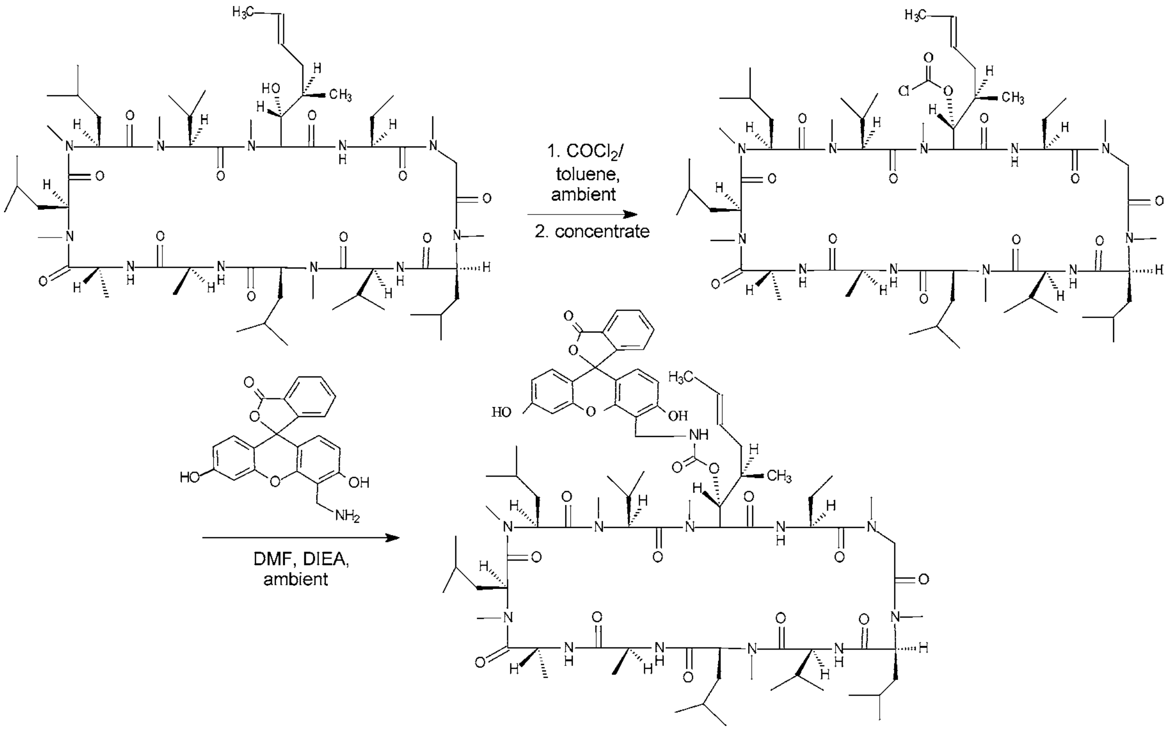

***Table 1.*** **Conditions, yields of cyclosporine tracer**

| entry | rxn time[a] (h) | additive(s)[a] | yield[b] (%) |
|---|---|---|---|
| 1 | 18 | – | 7 |
| 2 | 18 | $Et_3N$ (2X) | 26 |
| 3 | 36 | – | 37 |
| 4 | 168 | – | 50 |
| 5 | 192 | – | 48 |
| 6 | 168 | $Et_3N$ (2X) | 57 |

[a] For chloroformate formation. Standard conditions: 100 mg (84 umol) cyclosporine, phosgene in toluene (693 equiv), ambient, reaction time/additives in table. [b] Of conjugate.

particularly to mucous membranes, and should be used only in a properly operating fume hood with extreme caution) and the potential for internal pressure changes, a Teflon-lined screw-cap bottle was employed, rather than the typical round-bottom flask with a septum, cap, or glass-stopper closure. The reaction volume nearly filled the bottle, so that migration of phosgene into the headspace above the reaction was minimized.

Because of the combined corrosiveness and volatility of phosgene, increasing the temperature of a contained reaction was not viewed as a viable option. 4′-Aminomethylfluorescein was the required conjugate and was used in all reactions, obviating process optimization from a model substrate.

Initially, substitution of phosgene with the safer phosgene equivalents diphosgene, triphosgene, or carbonyldiimidazole (CDI) in the chloroformylation reaction using different solvents (THF, chloroform) was attempted. These reactions uniformly resulted in the production of little or no desired product, attributable to the steric congestion surrounding the MeBmt hydroxyl group.[13,16]

Solvents other than toluene were not added, to maintain the maximum phosgene concentration and hence the maximum reaction rate. However, subsequent trials using progressively longer reaction times demonstrated a substantial improvement in chloroformate production, with yields approaching 50% (see Table 1) at 168 h. Reaction times greater than 168 h lead to more highly colored chloroformates and diminished yields of conjugate. Independent trials also showed that amine bases could be added at the start of the reaction to improve yields by preventing undecapeptide hydrolysis or elimination of the hydroxyl group contained in the MeBmt residue due to the presence of HCl, a common impurity in phosgene solutions that have been exposed to moisture, as well as a direct product of the reaction. Triethylamine was selected in preference to other amines because of its volatility, since any excess reagent could be removed during chloroformate isolation by concentration, and because the product produced (which contained an unreactive amine salt) was a workable foam rather than a thick or sticky oil.

Combination of the two improvements nicely led to a synergistic yield improvement. Optimum conditions for production of the cyclosporine chloroformate were shown to be reaction of cyclosporine with 20% phosgene/toluene at ambient temperature in the presence of 2 equiv of triethylamine[18] for 168 h, followed by careful (via Rotovap attached to a water aspirator, followed by vacuum pump) concentration. Subsequent reaction with a solution of 4′-aminomethylfluorescein HCl and diisopropylethylamine in degassed dimethylformamide as solvent (required for label solubility, ambient temperature) provided the crude conjugate after concentration to remove the volatile materials (DMF). To determine an acceptable window of reaction times,

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(18) A stirrable precipitate, presumably triethylamine hydrochloride, formed when the triethylamine was injected. After 30 min, the precipitate had dissolved to provide a clear solution.

conjugations were varied from 18 to 72 h and demonstrated essentially equivalent product yields.

Purification of the cyclosporine−fluorescein conjugate was readily achieved using reversed phase HPLC, by which unreacted cyclosporine and 4′-aminomethylfluorescein were easily separated from the desired product using an isocratic acetonitrile-based, aqueous trifluoroacetic acid-modified solvent. The purification was easily scaled, due to the choice of Symmetry C8 as the solid phase, for which both analytical and preparative sizes are available.

Both the reaction and purification were scaled up 5-fold, producing equivalent yield and product purity without demonstrating any concentration effects or the process variability previously observed. For this cyclosporine−fluorescein conjugate, the yield was sufficient to meet product requirements for several years.

In conclusion, we have demonstrated that the synergistic improvements of extended reaction time and addition of amine base increased the yield of the cyclosporine chloroformylation process by 8-fold over what had been previously observed, resulting in a simple, reproducible process for the synthesis of a cyclosporine−fluorescein conjugate. Standardizing the chloroformylation process on a fixed amount of cyclosporine, a fixed volume of phosgene, and one size of reaction vessel eliminated the variation previously observed in the manufacturing area for a variable-batch process, permitting direct comparison of results and, ultimately, selection of the best process. Depending on the steric demand of the amine partner, this optimized process will prove useful for the synthesis of other conjugates as well.

## Experimental Section

Electrospray mass spectra (ESMS) were obtained on a PE Sciex instrument. Analytical HPLC was performed on a Waters Alliance system equipped with a Waters 2487 Dual Wavelength detector, and preparative HPLC was performed on a Waters preparative system. Elemental analysis was performed by Robertson Microlit. All chemicals are available commercially.

Cyclosporine (USP, 500 mg, 416 umol) was placed in an oven-dried, amber, ∼135-mL bottle equipped with a stirbar and a Teflon-lined screw cap. Phosgene solution (20%) in toluene was (130 mL, ∼600×) was carefully added via a graduated cylinder (CAUTION: Complete this process in a properly functioning, well-ventilated fume hood!), resulting in complete dissolution of the solid. Triethylamine (70 uL, 502 $\mu$mol) was injected.[18] The cap was screwed onto the bottle, and the clear, colorless solution was then stirred for 168 h at ambient temperature.

The solution and stirbar were poured into an oven-dried 100-mL round-bottom flask and stirred under aspirator vacuum for 10 min to remove excess phosgene (NOTE: by using water aspiration, phosgene is hydrolyzed to dilute hydrochloric acid). The solution was then concentrated to dryness on the Rotovap (∼30 Torr, <35°) using a water aspirator, and the residue was placed on a vacuum pump (<100 mTorr). After 90 min, 636 mg of a pale brown foam remained.

Anhydrous dimethylformamide (5 mL) was degassed for 10 min on a vacuum pump. 4′-Aminomethylfluorescein (305 mg, 626 umol) and diisopropylethylamine (320 $\mu$L, 1840 $\mu$mol) were dissolved in 2.0 mL of anhydrous degassed DMF and added to the crude chloroformate, followed by a 2.0 mL of DMF wash. The reaction was stirred for 3 h at ambient temperature. Analytical HPLC (Symmetry C8 4.6 mm × 150 mm column, eluting with 82% methanol/18% of 0.1% aqueous trifluoroacetic acid, 220/280 nm) showed one new major broad peak at 8.6 min (43.0% at 220 nm), attributable to tracer due to its 280 nm absorbance.

After stirring for 72 h, the reaction was concentrated on a Rotovap, and the remaining solution was purified directly by preparative HPLC on a 40 mm × 100 mm Symmetry C8 column, eluting with 81% methanol/19% of 0.1% aqueous trifluoroacetic acid (using 0.30 mL for each run, collecting from 0.15 au on the leading edge to the top of the peak to 0.15 au on the tailing edge). The combined fractions were concentrated on the Rotovap at ambient temperature until the methanol was removed and lyophilized. The product obtained was 438 mg of a bright-yellow solid: ESMS *m*/*z* 1591.4 ($M + H^+$); analytical HPLC (as above) 98.3% purity. Anal. Calcd for $C_{84}H_{124}N_{12}O_{18}\cdot 3.0H_2O\cdot 1.8CF_3COOH$: C 56.90, H 7.18, N 9.09, F 5.55. Found: C 56.96, H 7.43, N 9.07, F 5.40. Anal. (dried to constant weight) Calcd for $C_{84}H_{124}N_{12}O_{18}\cdot 0.8CF_3COOH$: C 61.16, H 7.48, N 10.00, F 2.71. Found: C 61.19, H 7.63, N 9.91, F 2.38.

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