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Synthesis of Cyclosporin A-Derived Affinity Reagents by Olefin Metathesis

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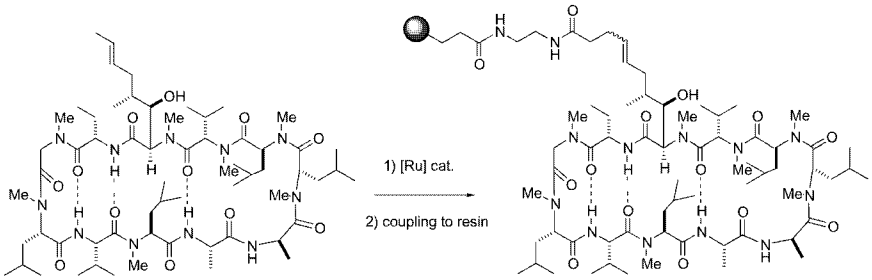
ABSTRACT



New affinity reagents were synthesized using alkene metathesis to directly modify the MeBmt side chain of cyclosporin A. The reagents were used to detect novel cyclophilins from cellular extracts.

The immunosuppressant cyclosporin A (CsA) **1** (Figure 1), known also by its trade name Sandimmune, is used to prevent organ rejection in transplant patients. CsA affects cellular signaling by first binding with high affinity to a cytosolic protein cyclophilin A.[1,2] The CsA−cyclophilin A complex binds to and inhibits the protein phosphatase activity of calcineurin,[3,4] which is an essential mediator of calcium signaling in T cells.[5] Cyclophilins comprise a superfamily of ubiquitously expressed cellular proteins with peptidyl-prolyl isomerase activity[6] and are thought to play roles in diverse cellular processes.[7] A complete understanding of the pharmacological effects of CsA in vivo necessitates the identification of all cyclophilins that are bound by the drug.

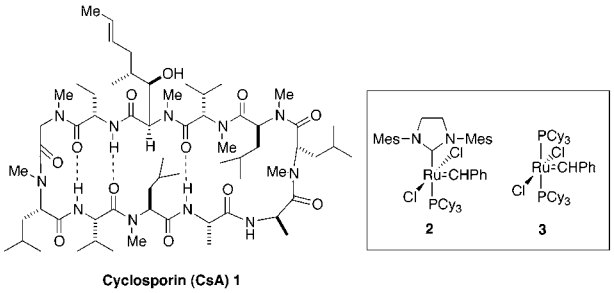


Figure 1. Cyclosporin A **1** and Metathesis Catalysts **2**−**3**.

To date, only a subset of cyclophilin-containing proteins have been isolated at the protein level. To identify new members

(1) Harding, M. W.; Rice, J.; Drugge, R. J. *Science* **1984**, *226*, 544−547.
(2) Fesik, S. W.; Gampe, R. T.; Holzman, T. F.; Egan, D. A.; Edalji, R.; Luly, J. R.; Simmer, R.; Helfrich, R.; Kishore, V.; Rich, D. H. *Science* **1990**, *250*, 1406−1409.
(3) Liu, J.; Farmer, J. D.; Lane, W. S.; Friedman, J.; Weissman, I.; Schreiber, S. L. *Cell* **1991**, *66*, 807−815.
(4) Liu, J.; Albers, M. W.; Wandless, T. J.; Luan, S.; Alberg, D. G.; Belshaw, P. J.; Cohen, P.; Mackintosh, C.; Klee, C. B.; Schreiber, S. L. *Biochemistry* **1992**, *31*, 3896−3901.
(5) Schreiber, S. L. *Science* **1991**, *251*, 283−287.

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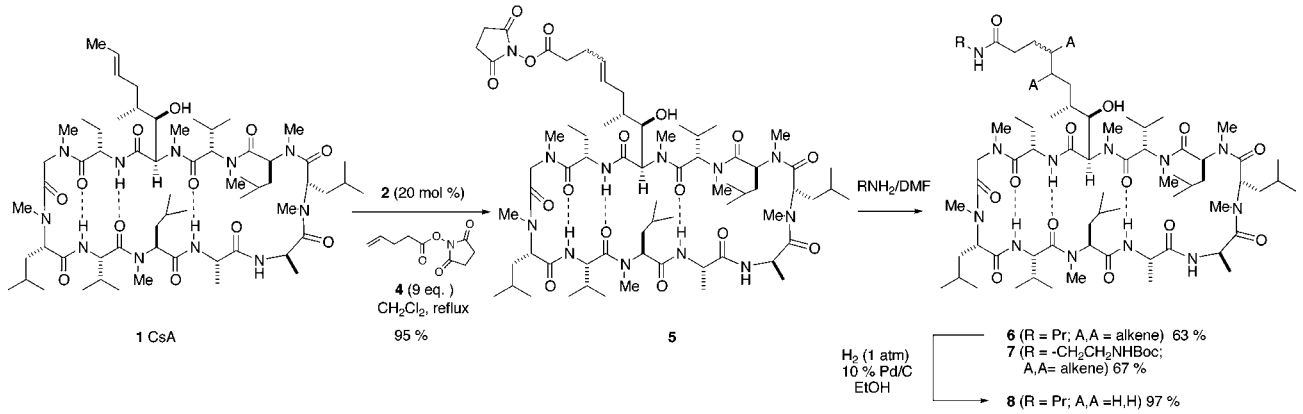
Scheme 1. Direct Modification of Cyclosporin A by Alkene Metathesis



of the cyclophilin superfamily, we set out to make an affinity matrix from readily available cyclosporin A using alkene cross metathesis. Because analogues of MeBmt that are modified on the butenyl portion of the molecule retain high binding affinity to cyclophilins,[8] this residue was chosen as the point of attachment to the solid support. In this communication, we report the synthesis of a novel CsA affinity reagent using alkene metathesis on the immunosuppressant CsA that permitted the detection of cyclophilins with higher molecular masses from cellular extracts.

Alkene metathesis has become a valuable synthetic method for the assembly of functionalized and substituted alkenes.[9] Recent developments in the Grubbs' ruthenium catalyst have led to increased reactivity without compromising functional group compatibility.[10] The new *N*-heterocyclic carbene-containing catalyst **2** has already been used in applications that were not possible with the first generation ruthenium catalyst **3**. Previously, the MeBmt side chain has been modified by a four-step reaction sequence that proceeds in 11−15% overall yield.[11] Dihydroxylation and oxidative cleavage of the alkene of MeBmt gives an aldehyde that can be reconstituted into the alkene by a nonstereoselective Wittig reaction with unstabilized and semistabilized ylides (10−40% yields). Another recent semisynthetic approach to cyclosporin analogues has been reported by Schreiber and co-workers for the synthesis of "bumped" cyclosporins by using a ring degradation/reconstitution approach.[12]

Direct alkene cross metathesis on the MeBmt side chain of CsA was achieved using an activated ester-bearing alkene as the reactive partner (Scheme 1). The choice of catalyst proved to be important in this conversion. Benzylidene **3** proved to be completely inefficient for cross metathesis. With catalyst **2**, it was found that CsA could be directly modified on the MeBmt alkene. Reaction took place at 45 °C using 9 equiv of **4** with 20 mol % catalyst.[13] Lower catalyst loadings were also used, but the reactions failed to run to completion. It is likely that the heating in the presence of the polypeptide results in some catalyst decomposition, which may explain why the relatively high catalyst loading is needed. This alkene metathesis was efficient using catalyst **2** and produced a 3:1 mixture of alkene isomers, which were assigned as *E* and *Z* isomers, respectively, on the basis of the relative ^{13}C chemical shifts of the alkene resonances.[14] The alkene mixture was inseparable by chromatography and was simply carried through the reaction sequence. This alkene metathesis is notable because of its direct installment of an activated ester onto the unprotected polypeptide.[15]

The active ester analogue **5** (Scheme 1) provides a ready means for the attachment to the amine-containing functionality. As expected, amines could be coupled to **5** in good yield. Hydrogenation of the alkene mixture of amide **6** was

(6) (a) Fischer, G.; Wittmann-Liebold, B.; Lang, K.; Kiefhaber, T.; Schmid, F. X. *Nature* **1989**, *337*, 476−478. (b) Takahashi, N.; Hayano, T.; Suzuki, M. *Nature* **1989**, *337*, 473−475.

(7) Walsh, C. T.; Zydowsky, L. D.; McKeon, F. D. *J. Biol. Chem.* **1992**, *267*, 13115−13118.

(8) Quesniaux, V. F. J.; Schreier, M. H.; Wenger, R. M.; Hiestand, P. C.; Harding, M. W.; Vanregenmortel, M. H. V. *Eur. J. Immunol.* **1987**, *17*, 1359−1365.

(9) (a) Trnka, T. M.; Grubbs, R. H. *Acc. Chem. Res.* **2001**, *34*, 18−29. (b) Chatterjee, A. K.; Grubbs, R. H. *Org. Lett.* **1999**, *1*, 1751−1753. (c) Chatterjee, A. K.; Morgan, J. P.; Scholl, M.; Grubbs, R. H. *J. Am. Chem. Soc.* **2000**, *122*, 3783−3784.

(10) (a) Scholl, M.; Ding, S.; Lee, C. W.; Grubbs, R. H. *Org. Lett.* **1999**, *1*, 953−956. (b) Sanford, M. S.; Ulman, M.; Grubbs, R. H. *J. Am. Chem. Soc.* **2001**, *123*, 749−750. (c) Sanford, M. S.; Love, J. A.; Grubbs, R. H. *J. Am. Chem. Soc.* **2001**, *123*, 6543−6554.

(11) Park, S. B.; Meier, G. P. *Tetrahedron Lett.* **1989**, *30*, 4215−4218.

(12) (a) Belshaw, P. J.; Schoepfer, J. G.; Liu, K. Q.; Morrison, K. L.; Schreiber, S. L. *Angew. Chem., Int. Edt. Engl.* **1995**, *34*, 2129−2132. (b) Belshaw, P. J.; Schreiber, S. L. *J. Am. Chem. Soc.* **1997**, *119*, 1805−1806.

(13) **Representative Experimental Procedure.** Into a flame-dried 50 mL Schlenk tube equipped with a magnetic stirbar and a reflux condensor was dissolved 200 mg (0.166 mmol, 1.0 equiv) of cyclosporin A, 295 mg (1.5 mmol, 9.0 equiv) of *N*-hydroxysuccinimidyl ester **4**, and 28 mg (33 μmol, 20 mol %) of **2** in 5.0 mL of CH_2Cl_2, and the solution was refluxed for 22 h. After cooling, the mixture was concentrated in vacuo (rotary evaporator) to give a solid that was purified by flash chromatography (elution with 15% CH_3CN−ethyl acetate) to give 200 mg (89% yield) of **5** as a white solid, mp 138−142 °C. Analytical TLC: R_f 0.20 (15% CH_3CN−ethyl acetate). ^1H NMR (500 MHz, $CDCl_3$, ppm): δ 8.11 (d, J = 10.0 Hz, 1H), 7.72 (d, J = 7.5 Hz, 1H), 7.49 (d, J = 8.5 Hz, 1H), 7.18 (d, J = 8.0 Hz, 1H), 5.70 (m, 1H), 5.57 (m, 1H), 5.47 (m, 1H), 5.37 (m, 1H), 5.32 (m, 1H), 5.11−4.97 (m, 4H), 4.83 (m, 1H), 4.72 (m, 1H), 4.66 (m, 1H), 4.52 (m, 1H), 3.88−3.80 (m, 2H), 3.50 (s, 3H), 3.40 (s, 3H), 3.27 (s, 3H), 3.12 (s, 3H), 3.11 (s, 3H), 2.84 (m, 4H), 2.70 (s, 3H), 2.68 (s, 3H), 2.43−0.71 (m, 72H). FT-IR (film, cm^{-1}): 3478, 3329, 2951, 2866, 1737, 1630, 1518, 1215, 1076. FAB-MS: molecular ion calcd for $C_{68}H_{116}N_{12}O_{16}$ 1357, found 1358 (M + H).

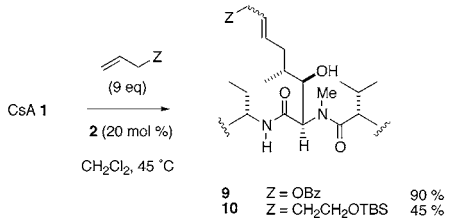
(14) Olefinic carbons of the minor alkene isomer appeared at δ 130.6 and 126.8, and the isomer was assigned as **5*Z***; the major alkene isomer gave resonances at δ 131.2 and 128.2 and was assigned as **5*E***.

(15) Miller, S. J.; Blackwell, H. E.; Grubbs, R. H. *J. Am. Chem. Soc.* **1996**, *118*, 9606−9614.

uneventful and provided the dihydro MeBmt analogue **8** in 99% yield. For this conversion, heterogeneous catalysis by 10% Pd/C (H_2 balloon) proved to be more effective than 5% Rh on alumina. This demonstrates that hydrogenation could be used when necessary to reduce the alkene mixtures. On the basis of literature precedent, hydrogenation of the alkene of CsA would not be expected to affect binding to cyclophilin A.[6]

Different MeBmt derivatives were synthesized by alkene metathesis using terminal alkenes. As the basis for selecting alkenes, we considered those that possess functional handles that could be used to prepare conjugates. Because of the mildness of the ruthenium carbene catalysts, it is imaginable that more complex alkenes could be introduced onto alkene-containing biomolecules by the metathesis approach. Treatment of CsA with 9 equiv of allyl benzoate or 5-(*tert*-butyldimethylsilyloxy)-1-pentene gave the cross metathesis products in good yields (Scheme 2). The ratio of *E* and *Z*

Scheme 2. MeBmt Modification with 1-Alkenes

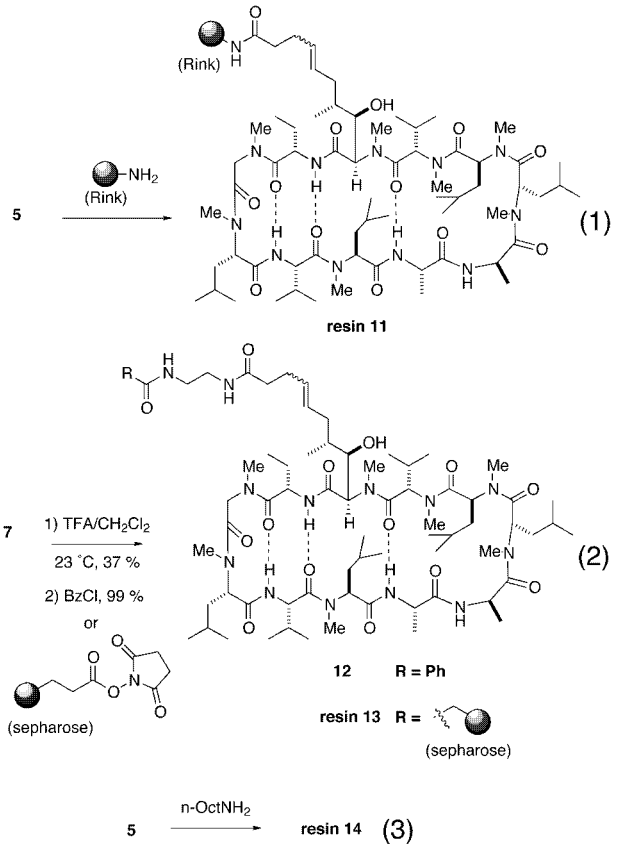


alkene isomers was determined to be 2:1 by ^{1}H NMR. With an excess of the 1-alkene, no homodimerization of CsA was observed.

The cross metathesis reactions are not limited to terminal alkenes as the reactive partner. In the absence of a 1-alkene, **1** undergoes a slow homometathesis with concomitant loss of propylene gas.[16] Heating CsA with **2** in dichloromethane for 48 h resulted in 50% conversion (reversed-phase HPLC) of **1** to its metathetical homodimer (not shown).[17] The CsA dimers might be useful as bivalent ligands that could be used to regulate biologic processes initiated by protein dimerization, a concept pioneered by Schreiber and Crabtree.[18]

Resins were prepared from active ester **5**. Fmoc-Protected Rink resin was deprotected with 20% piperidine, washed, and then shaken with the CsA-derived active ester **5** to produce modified Rink resin **11** (eq 1, Scheme 3). The resin was used directly in an attempt to bind cyclophilins from a cellular extract. However, resin **11** failed to pull down any cyclophilins, possibly due to poor swelling properties of this hydrophobic resin in aqueous buffer. Commercially available sepharose-based resins are used to bind proteins in aqueous environments and are available with activated carboxylic esters on the surface. To couple to carboxylic activated resins, the CsA molecules obtained through metathesis should possess amino groups for coupling. Succinimidyl ester **5** was coupled to mono *N*-Boc-ethylenediamine in 67% yield isolated after column chromatography. Deprotection of the *t*-Boc group (1:1 v/v TFA/CH_2Cl_2, 0 °C) of **7** gave the

Scheme 3. Resin Modification by CsA



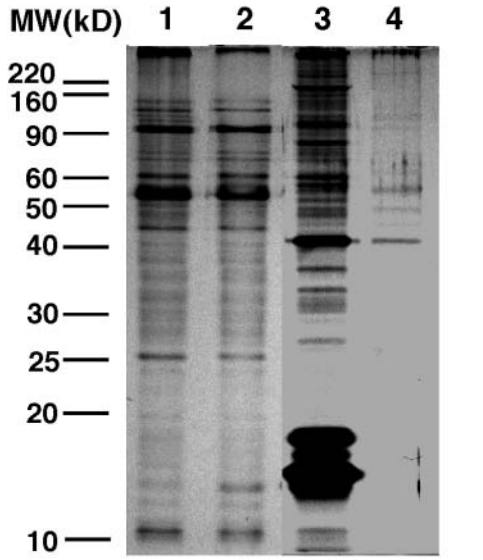
primary free amine after extractive workup in 37% yield; the amine was immediately benzoylated (BzCl, Et_3N, rt, 12 h) to give **12** in 99% isolated yield (eq 2). After demonstration of the presence of the free amine, **7** was deprotected and coupled onto Sepharose resin, prepared in the usual way, to give the desired CsA-modified resin **13**. The unreacted, surface-accessible active esters on the polymer were capped by subsequent treatment with ethanolamine.[1] In addition, a control resin was prepared by coupling *n*-octylamine to activated sepharose to provide resin **14** (eq 3) of the same surface loading as resin **13**. Both the resin **13** and the control resin **14** were used to test for protein binding.

The results of the CsA affinity binding experiments are shown in Figure 2. Cell lysates prepared from a colon cancer cell line RKO were incubated with the resins at 4 °C for 1 h. The resins were then washed three times with the binding buffer (20 mM Tris HCl, pH 7.4, 100 mM KCl, 0.2% Triton X-100, 2 μg/mL each of leupeptin, aprotinin, and soybean trypsin inhibitor) with or without 10 μM of sanglifehrin A, another natural product with a high affinity for cyclophilins.[19] The proteins retained on the resins were dissociated into a gel-loading buffer containing sodium dodecyl sulfate in

(16) Related dimerization of FK506: Diver, S. T.; Schreiber, S. L. *J. Am. Chem. Soc.* **1997**, *119*, 5106−5109.

(17) The homodimer was obtained as an *E*/*Z* mixture and was contaminated with unreacted cyclosporin.

(18) Spencer, D. M.; Wandless, T. J.; Schreiber, S. L.; Crabtree, G. R. *Science* **1993**, *262*, 1019−1024.



1. Control Sepharose
2. Control Sepharose + Sanglifehrin A
3. CsA-Sepharose
4. CsA-Sepharose + Sanglifehrin A

Figure 2. Detection of cyclophilins using CsA−sepharose **13**. Affinity beads (20 μL) were mixed with 40 μL of SDS sample buffer, followed by heating in boiling water for 3 min. Each supernatant (20 μL) was subjected to 12% denaturing SDS−PAGE, and the gels were stained with Coomassie blue. Lane 1: proteins bound by the control resin **14**. Lane 2: proteins bound by control resin **14** with sanglifehrin A (10 μM). Lane 3: proteins bound by resin **13**. Lane 4 is the same as lane 3, except that sanglifehrin A (10 μM) was present in the washing buffer.

boiling water, followed by gel electrophoresis. As expected, the control resin **14** showed no binding to any known cyclophilins (Figure 2, lane 1). In contrast, resin **13** showed binding to a number of cyclophilins (Figure 2, lane 3). The bands with strongest intensity include the known cyclophilins A, B, and cyclophin-40, as judged by their molecular weights of 18, 19, and 40 kD, respectively. In addition, several higher molecular weight proteins were observed with resin **13** that have not been previously identified. They possess apparent molecular masses of about 58, 60, 80, 95, 180 kD, respectively. It is likely that binding of these proteins to resin **13** is mediated by cyclophilin domains, as protein binding to the resin is sensitive to competition by sanglifehrin A (Figure 2, lane 4). In contrast, the nonspecific binding of proteins to the control sepharose was not competed by the same concentration of sanglifehrin A (Figure 2, lane 2 vs lane 1).

In summary, the preparation of a new cyclosporin A-derived affinity reagents for protein binding using cross alkene metathesis is described. The alkene metathesis strategy may prove to be useful for the attachment of small molecules to a solid support or for identification of receptor proteins for small molecule ligands. By using the reactive type II benzylidene ruthenium carbene **2**, a variety of alkenes, including active esters, can be introduced into the 1-position of the cyclic undecapeptide. The activated esters could be converted efficiently into resin-bound cyclosporins, which were used for the detection of novel cyclophilins from cell lysates. Isolation and identification of those high molecular mass cyclophilins may shed new light on the mechanism of action of CsA.

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Supporting Information Available: Detailed experimental procedures for the preparation of compounds **5**−**13**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(19) (a) Sanglier, J. J.; Quesniaux, V.; Fehr, T.; Hofmann, H.; Mahnke, M.; Memmert, K.; Schuler, W.; Zenke, G.; Gschwind, L.; Maurer, C.; Schilling, W. *J. Antibiot.* **1999**, *52*, 466−473. (b) Fehr, T.; Kallen, J.; Oberer, L.; Sanglier, J. J.; Schilling, W. *J. Antibiot.* **1999**, *52*, 474−479.