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GLYCOL AND OLEFIN ELECTROPHORIC RELEASE TAGS

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Abstract - An "electrophoric release tag" is a molecular labeling reagent which comprises three covalently-connected groups: electrophore/release/ reactivity. This reagent is first covalently attached *via* its reactivity group to a substance of interest. The subsequent release tag-substance conjugate is detected by chemically cleaving the release group within the release tag, liberating the electrophore for eventual detection by electron capture in the gas phase. Here electrophoric release tags are advanced by the preparation of three similar tags having a glycol release group. One of the glycol tags, *N*-(pentafluorobenzoyl)-4-piperidylidenyl-acetic acid glycol, is attached to glycine methyl ester as a model analyte. The resulting conjugate is cleaved within 5 minutes by aqueous periodate at room temperature, releasing the electrophore *N*-(pentafluorobenzoyl)-4-piperidone, for detection. Also a tag with an olefin release group is prepared: *m*-(pentafluorobenzyloxy)-*E*-3-methylcinnamoyl chloride. The latter reagent is attached to thymidine as a model analyte and a subsequent methylated conjugate is cleaved within 1 hr by aqueous permanganate/ periodate, releasing the electrophore *m*-(pentafluorobenzyloxy)-acetophenone for detection.

An electrophoric release tag is a molecular labeling reagent which comprises three covalently-connected groups: electrophore/ release/ reactivity.[1] Once this tag is attached *via* its reactivity group to a substance of interest, forming a release tag-substance conjugate, the latter can be detected by cleaving the release group within the release tag, followed by determination of the released electrophore. Electrophoric release tags are intended to be used as signal groups in chemical analysis in the same way radioisotopes and fluorophores are used.

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An electrophore is a molecule that avidly captures a thermal electron in the gas phase. It thereby is readily detected by gas chromatography with electron capture detection (GC-ECD) or a related technique. Electrophores are attractive as signal groups in chemical analysis because they can be chemically and physically stable, relatively small in size, determined at trace levels (e.g. $< 10^{-18}$ mole[2]), and available in numerous molecular forms. The latter property of electrophores is particularly unique: taking both practical and theoretical considerations into account, no other type of signal group can offer the multiplicity available from electrophores. This aspect has been elaborated elsewhere.[1]

The usefulness of electrophores as signal groups has been limited by the necessity of determining them in the gas phase. Unfortunately, many substances are too nonvolatile or thermally labile for this type of determination. The purpose of electrophoric release tags is to allow electrophores to be employed as labels for such substances, since only the released electrophore, not the electrophore-substance conjugate, is brought into the gas phase.

The first electrophoric release tag, N-pentafluorobenzoyl-methionyl-glycine-N-hydroxysuccinimide ester, was used to determine the analyte thyroxin in serum. An electrophore thyroxin conjugate was formed which was subsequently detected, after purification, by cleavage at its methionylamide release group with cyanogen bromide to release the electrophore, N-pentafluorobenzoylhomoserine lactone, for extraction and quantitation by GC-ECD.[1] Unfortunately, cyanogen bromide is toxic, the release reaction took 1 hr at 70°C, and a lactone is somewhat polar as a solute for GC.

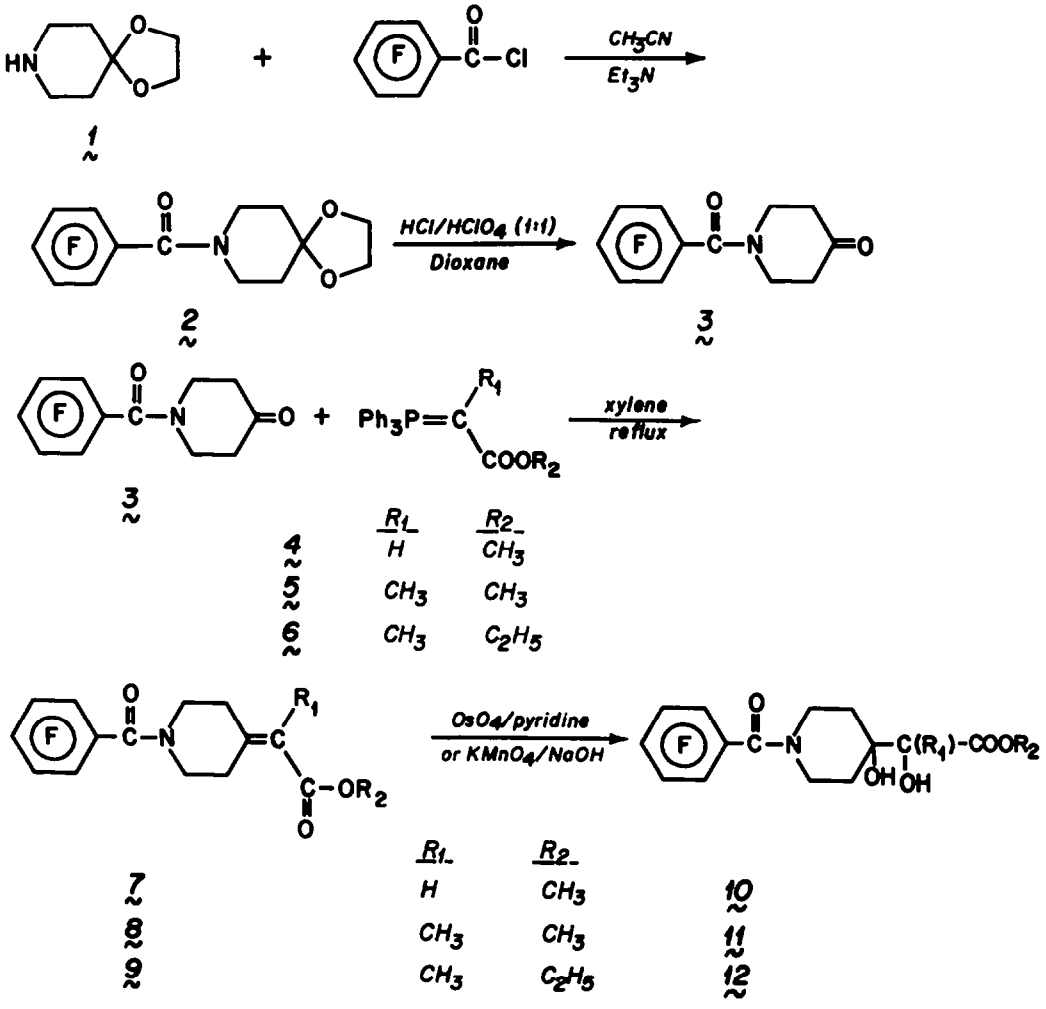
This paper reports the preparation and properties of two additional types of electrophoric release tags based primarily on the nature of the release group: glycol and olefin.

RESULTS AND DISCUSSION

I. Glycol Release Tag

Electrophoric release tags are intended to be especially useful as labels for biological macromolecules such as antibodies and DNA probes. A successful tag should therefore be water soluble and susceptible to

cleavage under aqueous conditions. Also the electrophoric signal group should be released in a volatile form by the cleavage reaction. A glycol release group possessing a tertiary hydroxyl group on the side connecting to the signal group meets these criteria. It is well-known that glycols are efficiently cleaved under aqueous conditions by periodate.[3] A ketone results when a compound possessing a tertiary hydroxyl is cleaved by periodate, and ketones are good solutes for GC. Taking this into account along with the good GC-ECD properties of a pentafluorobenzamide group,[2] we prepared the glycols 10-12 as release tag precursors as shown here.



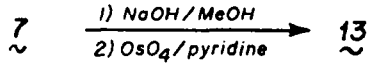
Acylation of 1 was carried out at 0°C in the presence of Et_3N as a catalyst. Three different solvents were tried, benzene, DMF and acetonitrile. The highest yield (97%) was obtained using the latter solvent. Hydrolysis of the ketal 2 using concentrated $HClO_4$/HCl (1:1) in dioxane gave the piperidone derivative 3. The electrophoric ketone 3 was converted to olefins 7, 8 and 9 via a Wittig reaction, utilizing three different phosphoranes 4, 5 and 6. Less successful for this olefin synthesis was a malonic acid synthesis[4] which gave a lower yield and more side products. Phosporanes 5 and 6 relative to 4 were sluggish in this reaction due to the presence of an α-methyl group. Probably this methyl sterically interacts with the nearby protons on the piperidone ring of 4 during the formation of the intermediate betaine. The diol release group in compounds 10, 11 and 12 was formed by treating 7, 8 and 9, respectively, at 0°C with osmium tetroxide and pyridine in dry tetrahydrofuran,[5] or with dilute $KMnO_4$ and NaOH in t-butanol/acetone.[6]

Formation of the Carboxyl Reactivity Group

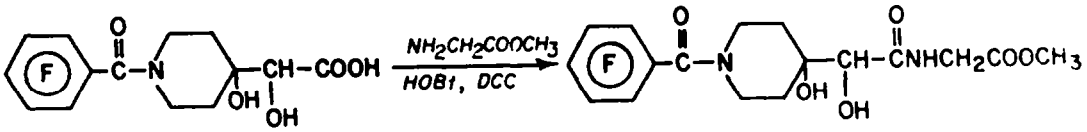
The diol esters 10 and 11 were hydrolyzed by refluxing in methanolic sodium hydroxide for 30 minutes to give the corresponding diol acids 13 and 14. The diol acid 13 was also prepared by hydrolysis of the olefinic ester 7 followed by oxidative dihydroxylation as before.

13 $R_1 = H$

14 $R_1 = CH_3$



We demonstrated the labeling reactivity of the release tag by attaching it to glycine methyl ester as a model target substance.



13 15

The diol acid 13 was activated with dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBT) and reacted with glycine methyl ester[7] in chloroform in presence of triethylamine, yielding the conjugate 15.

Release Tag Cleavage

As intended, the release tag-glycine methyl ester product 15 undergoes facile cleavage with aqueous periodate to form ketone 3 as a released electrophoric signal group in a quantitative yield (determined by HPLC). The other glycols react the same way.

Rate of Release

The best release tag, other considerations aside, will undergo the fastest release when subjected to the cleavage reaction. In order to optimize the conditions for this cleavage reaction, we obtained the data shown in Table I. As seen, the rate of the reaction is examined as a function of glycol structure and reaction conditions. Consistent with the complex nature of glycol cleavage by periodate,[2,8] a wide variation in cleavage rates is observed. Faster release, a desirable property for a release tag, is promoted mainly by R_1 = H (10,13,15) instead of CH_3 (11,12); a carboxyl (13,14) or amide (15) instead of an ester (10,11,12) group on the glycol; methanol > acetonitrile > dioxane as an organic cosolvent; a higher percentage of water; and a higher temperature. No doubt release is faster with R_1 = H as opposed to CH_3 because the latter group sterically interacts with a nearby CH_2 group of the piperidyl ring when the periodate forms a cyclic intermediate[9] with the glycol moiety.

Table I. Rates of glycol cleavage to form the ketone 3.

Glycol	Reaction conditions for cleavage with $NaIO_4$[a]		Rate constant (sec^{-1} x 10^3) at 23°C (and 60°C)[b]	
10	CH_3CN/H_2O,	50/50	3.3	(5.6)
11	"	25/75	0.048	
	"	50/50	0.011	(0.076)
	"	75/25	0.0024[c]	
12	"	25/75	0.037	
	"	50/50	0.015	(0.053)
	"	75/25	0.0026[c]	
13	"	50/50	9.2	
14	"	50/50	1.4	
15	"	50/50	6.9	
10	Dioxane/H_2O,	50/50	2.1	
	CH_3OH/H_2O,	50/50	8.9	
	CH_3CN/0.11 M, aq. Na phosphate pH 7	5/95	45	
	CH_3CN/aq. 0.23 M $NaNO_3$	5/95	50[d]	
	CH_3CN/aq. 3.2 M urea	5/95	67[e]	

Footnotes for Table I

[a]At time zero, 0.2 mL of 0.5 M aqueous sodium periodate solution was added rapidly followed by vortexing to 0.2 mL of a 2.5 mM solution of the glycol in the given solution to give the final composition shown, v/v. The final concentration for the glycol was 1.25 mM except as noted. [b]Aliquots of 0.01 mL of the reaction mixture were removed as a function of time and injected directly onto a reversed phase HPLC column to both stop the reaction and simultaneously determine the amounts of starting glycol and product ketone. These two compounds had equivalent responses by HPLC with absorbance detection at 254 nm. No other peaks were seen, and the amount of ketone which formed corresponded to the amount of glycol which disappeared. Consistent with pseudo first-order kinetics, plots of ln(glycol peak area/ketone + glycol peak area) *vs* time were linear, and the absolute value of the correlation coefficient for each of these lines was greater than 0.98 in most cases and always above 0.93. [c]In these experiments 0.1 mL of aqueous periodate was added to 0.3 mL of the glycol in acetonitrile giving a final glycol concentration of 1.8 mM. [d]This reaction was nearly complete by the time of the first HPLC measurement, allowing only a single kinetic point to be obtained. The experiment was repeated three times, and the standard deviation for the mean value shown was 0.007. [e]Same as footnote d, with a standard deviation of 5.1.

A variety of aqueous conditions are anticipated for applications of release tags. It is important then that compound 10 as a test case maintains a rapid release (a rate constant of 50 x 10^{-3} sec^{-1} corresponds to a reaction half-life of 14 sec) irrespective of the apparent pH (5.0 to 7.0), ionic strength, and presence or absence of organic solvent or 3.2 M urea in the reaction mixture.

Conclusion for the Glycol Release Tag

4-Piperidylidenyl-glycol is an attractive release group for electrophoric release tags. Its structural features and its release reaction are compatible with aqueous as well as aqueous/organic solvent conditions. Sodium periodate, the cleavage reagent, is nontoxic and stable, and the release reaction only requires a few minutes at room temperature. The released ketone is a stable, nonpolar structure for organic extraction and determination by GC-ECD.[10]

II. Olefin Release Tag

A glycol release tag, since it possesses free hydroxy groups, is not suitable for labeling a hydroxyl group on a target substance. In order to overcome this limitation, we developed a release tag having an olefin release group. In particular we prepared an olefin release tag which was essentially an electrophore-substituted cinnamyl chloride. Such a tag was expected to give a relatively stable ester linkage to a hydroxyl-containing target substance. This is because we had observed previously that a deoxynucleoside which was O-substituted on the sugar moiety with cinnamyl groups was resistant to aqueous hydrolysis.[11] First we prepared the electrophoric acetophenones 16a-c shown in Fig. 1. As will be presented, the release tag derived from 16a is ultimately the most successful.

Olefin Ester Synthesis

The ketones 16a-c, as shown in Fig. 1, were subjected to either a Wittig reaction[12] with methyl(triphenylphosphoranylidene)acetate or to a Horner-Emmon's reaction[13] with methyldiethylphosphonoacetate. The desired E and Z olefins 17a-c and 18a-c were obtained along with side

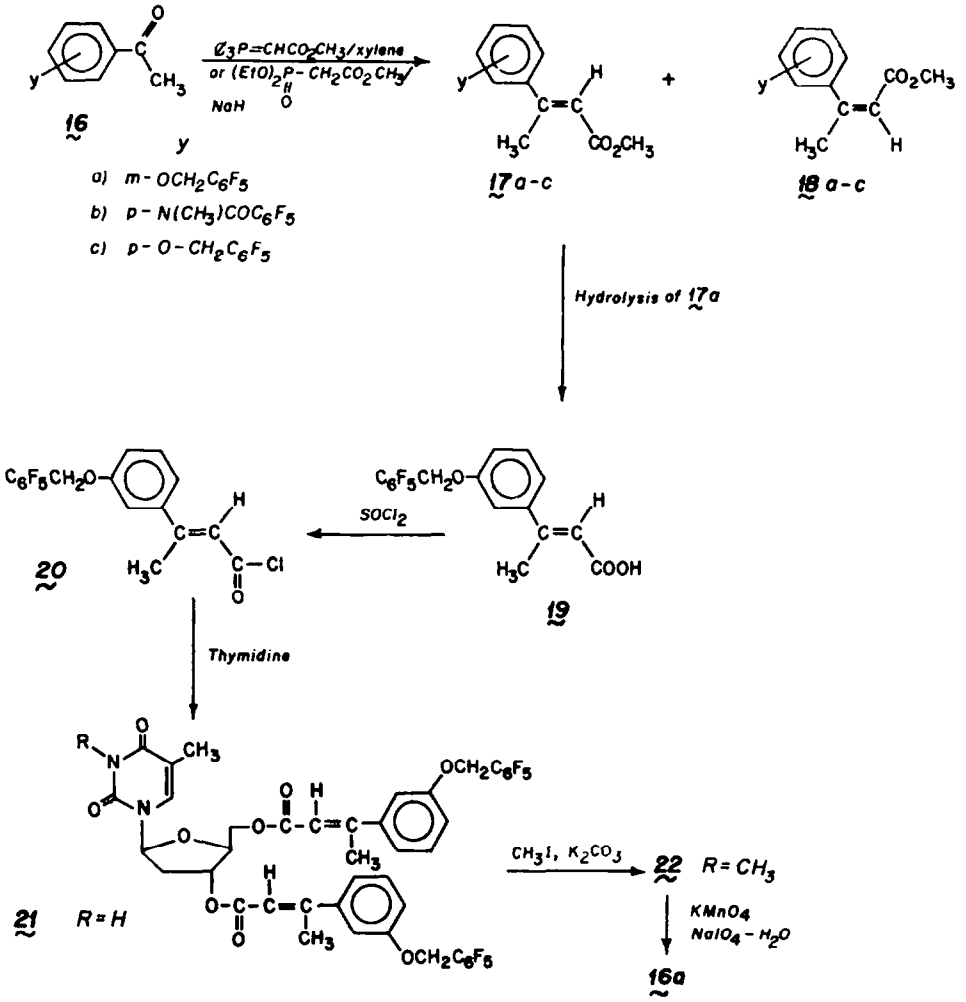


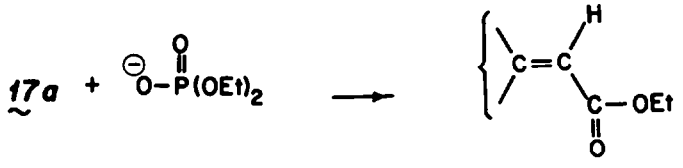
Fig. 1

Synthesis and Testing of an Olefin Release Tag

products. This conversion was studied in most detail starting with the ketone 16a.

For 16a, the Horner-Emmon's reaction is most successful, yielding 71% of the E olefin 17a after flash chromatography. The yield of the corresponding Z-olefin 18a is 3%. This result is consistent with the report that phosphonate carbanions react with aldehydes or ketones to give predominantly E vs Z olefins.[14]

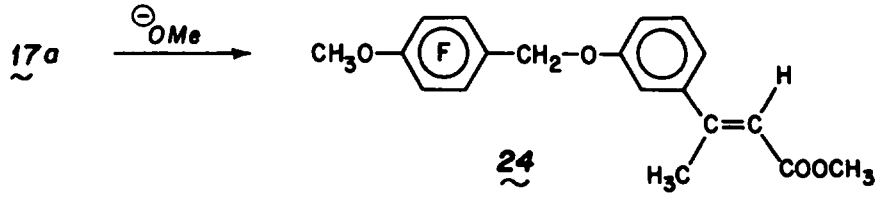
Two unexpected side products are isolated and identified from the Horner-Emmon's reaction on 16a. The first one is the E olefin ethyl ester 23. Apparently this product arises by a trans-esterification of 17a as follows:



23

In agreement with this mechanism, we obtain the ethyl ester 23 (in a quantitative yield) when we independently react the methyl ester 17a with diethylphosphate and sodium hydride in tetrahydrorfuran.

The other side product, 24, arises by nucleophilic displacement of the p-fluorine atom on the pentafluorophenyl ring by the methoxide released in the above trans-esterification reaction.



24

We independently prepared 24 by reacting 17a with sodium methoxide in methanol at room temperature. The two preparations of 24 are the same in all respects including identical ^{19}F NMR spectra. The latter data also confirms that the methoxy group is located at the para position. It is well established that nucleophilic substitution on pentafluorophenyl compounds takes place at this position.[15]

The ketones 16b and 16c behave similarly to 16a when subjected to the Horner-Emmon's reaction, including the relative amounts and yields of the corresponding E and Z olefins.

Hydrolysis of the Ester Group and Conversion to an Acid Chloride.

The methyl ester 17a is successfully hydrolyzed to the corresponding acid 19 using a two-phase reaction involving t-butanol and aqueous potassium hydroxide (5 N) with refluxing for 3 hours. This reaction condition was employed after it was found that exposure of 17a to aqueous methanolic sodium hydroxide largely forms the corresponding acid of 24, in which a para-methoxy group is present. Therefore we hydrolyzed 17a with t-butanol and aqueous potassium hydroxide to avoid nucleophilic displacement of the p-fluorine atom on 17a. All attempts to hydrolyze 17b and 17c gave either no hydrolysis or a low yield of the desired acids due to the formation of many side products. Thus 17b and 17c were not investigated further.

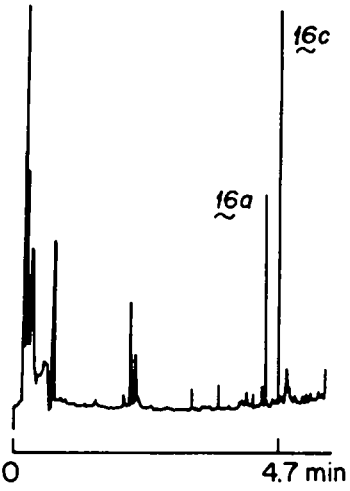
The acid 19 was converted to the acid chloride 20 using thionyl chloride in benzene. This solution was evaporated to an oil which was used immediately.

Preparation and Detection of Release Tag-Thymidine

To demonstrate the labeling, release, and detection properties of the olefinic release tag 20 towards a hydroxyl-bearing analyte, we reacted it with thymidine as shown in Fig. 1. (Prior to this we had reacted 20 with isopropanol and characterized the resulting isopropyl ester 25. We have similarly reacted 20 with glycine methyl ester.) This nucleoside, which possesses both a primary and secondary hydroxyl group, is of interest as a model analyte in our work using electrophore labeling to quantify chemical damage to DNA.[16]

We were pleased to observe that thymidine is quantitatively labeled on both hydroxyls by reaction with 20. Further reaction of the product with methyl iodide (Fig. 1) methylates it at N3, yielding 22. This facilitated the direct characterization of 22 by GC and MS. As intended, 22 is hydrolytically stable under moderate aqueous conditions. It is unchanged, based on TLC, when incubated in aqueous buffer for 24 hours either at pH 4 or 8. Assays involving release tags would generally use comparable or milder aqueous conditions.

Compound 22 yields the volatile,electrophoric ketone 16a as a released signal group when subjected to oxidative cleavage with aqueous potassium permanganate/sodium periodate.[17]. Once the conditions of this reaction were optimized, as little as 10 picograms of 22 could be detected by the sequence: oxidative cleavage (1 hr at room temperature), extraction, and GC-ECD, giving a GC chromatogram such as that shown in Fig. 2. The second peak, after that of 16a, is an internal standard 16c added prior to the oxidation reaction. The absolute yield of 16a is 70% based on external standardization.



Conclusion for the Olefin Release Tag

A successful olefin tag has been prepared for labeling of a substance having a reactive hydroxyl group. Because the olefin component of this tag is part of a cinnamyl moiety, the tag forms a relatively stable ester linkage to a target substance, contributing to its potential usefulness.

EXPERIMENTAL SECTION

^{1}H NMR spectra were obtained on a Varian XL-300FT or Varian T-60A instrument. The chemical shift data are reported in parts per million (δ) relative to tetramethylsilane. Infrared spectra were taken on Perkin-Elmer Model X99 spectrophotometer and the absorptions are reported in wave numbers (cm^{-1}). Mass spectra were obtained on either a Nuclide 12-90-G instrument or, for high resolution, on a C.E.C. 21-110B. Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Silica TLC plates from ANALTECH were used to monitor reactions. Silica gel (230-400 mesh) used for flash chromatography was obtained from Sigma. All solutions were v/v unless indicated otherwise. All drying was anhydrous Na_2SO_4 unless indicated otherwise.

N-(Pentafluorobenzoyl)-4-piperidone ethylene ketal (2). To 4-Piperidone ethylene ketal, 1 (2.04 g, 14.5 mmol) in dry CH_3CN (50mL) containing Et_3N (3mL) at 0°C was added pentafluorobenzoyl chloride (3.36 g, 14.5 mmol)

dropwise with stirring, keeping 0-5°C. After stirring at RT for 15 min and dilution with EtoAc (60 mL), the organic layer was washed with 10% HCl, 10% $NaHCO_3$, and H_2O, followed by drying. Evap afforded a white solid which was recryst from hexane/acetone to yield colorless crystals of 2 (4.7 g, 97%); mp 99-101°C; IR (film): 2960, 1820, 1660, and 1510 cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.75 and 1.86 (two t, 4H, 2 CH_2 adjacent to the ketal), 3.42 and 3.92 (two t, 4H, 2 CH_2 adjacent to the N_2), and 4.00 (s, 4H, ethylene ketal); MS, m/z 343 (M^+), 195 and 167.

N-(Pentafluorobenzoyl)-4-piperidone (3). A solution of 2 (4.17 g, 13.7 mmol) in dioxane (50 mL) was treated with conc HCl and $HClO_4$ (12 mL, 1:1, v/v), refluxed for 4 hr, and EtoAc was added. The organic layer was washed with 10% $NaHCO_3$ and H_2O and dried. Evap afforded a white solid which was purified by flash chrom on silica gel (3:7 EtoAc/hexane) yielding 3 (3.4 g. 85%); mp 124-125°C; IR (film): 1740 and 1650 cm^{-1}; ^1H NMR ($CDCl_3$): δ 2.50 and 2.63 (two t of 2 CH_2 adjacent to the carbonyl), 3.67 and 4.09 (two t, 2 CH_2 adjacent to the N_2); MS, m/z 293 (M^+), 195 and 167.

Methyl-[N-(pentafluorobenzoyl)-4-piperidylidenyl]acetate (7). The piperidone derivative 3 (2.93 g, 10 mmol) and methyl(triphenyl- phosphoranylidene)acetate 4 (3.35 g, 10 mmol) in xylene (100 mL) was refluxed under N_2 for 3 hr. Evap (vac) afforded a dark yellow solid for flash chrom (3:7 EtoAc/hexane), yielding a white solid (3.35 g, 96%); mp 112-113°C; IR (film): 2990, 2930, 1705, 1650 and 1620 cm^{-1}; ^{1}NMR ($CDCl_3$):δ 2.36, 2.47, 3.11, 3.02 (four sets of t, 4H, 2 CH_2 adjacent to the double bond), 3.69, 3.72 (two s, 3H, methyl ester), 3.40, 3.42, 3.86 and 3.89 (four t, 4H, 2 CH_2 adjacent to the N_2), and 5.77, 5.81 (two s, 1H, vinylic proton); MS, m/z 349 (M+), 318, 290, 195 and 167.

Methyl-2-[pentafluorobenzoyl)-4'-piperidylidenyl]propionate (8). The piperidone derivative, 3 (2 g, 6.8 mmol) and methyl-2-(triphenyl-phosphoranylidene)propionate 5 (2.37 g, 6.8 mmol) in xylene (120 mL) was refluxed for 48 hr. Evap (vac) afforded a yellow solid for flash chrom (2:8 EtoAc/hexane), to yielding a white solid which was recryst from hexane/acetone; (1.6 g, 65%); mp 91-92°C; IR (KBr): 2950, 2870, 1715 and 1650 cm^{-1}; ^1H NMR ($CDCl_3$):δ 2.02 and 2.03 (two s, 3H, alkyl CH_3), 2.50, 2.55, 2.73 and 2.82 (four t, 4H, 2 CH_2 adjacent to the N_2),

3.74 and 3.78 (two s, 3H, methyl ester); MS, m/z 363 (M+), 332, 331, 304, 303, 195 and 167.

Ethyl-2-[N-(pentafluorobenzoyl)-4'-piperidylidenyl]propionate (9). The piperidone derivative, 3 (1.75 g, 5.96 mmol) and ethyl-2-(triphenylphosphoranylidene)propionate 6 (2.1 g, 5.96 mmol) in xylene (70 mL) was refluxed under N_2 for 72 hr. Evap (vac) afforded a yellow oil for flash chrom (3:7 EtoAc/hexane) to give a white solid (1.2 g, 53%); mp 76°C; IR (film): 2980, 2925, 2865, 1710, and 1660 cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.31 and 1.34 (two t, 3H CH_3 of ethyl ester), 1.90 and 1.96 (two s, 3H, alkyl CH_3), 2.50, 2.55, 2.73 and 2.82 (four t, 4H, 2 CH_2 adjacent to the N_2), 4.20 and 4.25 (two q, 2H, CH_2 of ethyl ester); MS, m/z 377 (M+), 348, 332, 331, 304, 195 and 167. Anal. calcd for $C_{17}H_{16}F_5NO_3$: C, 54.11; H, 4.27; N, 3.71. Found: C, 53.97; H, 4.35; N, 3.67.

Methyl-[N-(pentafluorobenzoyl)-4-piperidylidenyl]acetate glycol (10). The α,β-unsaturated ester, 7 (1.25 g, 3.58 mmol) was hydroxylated with osmium tetroxide (1.0 g, 3.91 mmol) and pyridine (5 mL) in dry tetrahydrofuran (26 mL). After stirring under N_2 at RT for 3 hr, a solution of 10% sodium bisulfite (30 mL) was added, and the reaction mixture was stirred vigorously for 90 min. EtoAc was added, and the aqueous layer was extracted 4 times wit EtoAc. The combined organic extracts were washed with 10% HCl, 10% $NaHCO_3$ and H_2O, and then dried. Evap afforded a white solid that was recryst from methanol/hexane to yield colorless needles (1.27 g, 92%), one spot on TLC (1:1 EtoAc/hexane); mp 115-117°C; IR(KBr): 3480, 3270, 1730, 1640 cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.77 (br m, 4H, 2 CH_2 adjacent to the glycol) 3.37 (br m, 4H, 2 CH_2 adjacent to the N_2), 3.72 (s, 3H, methyl ester), 3.93 (br s, 1H, CH) 4.37 and 4.58 (br s, 2H, 2 OH of the glycol); MS, m/z 383 (M+), 294, 195 and 167.

Methyl-2-[N-(pentafluorobenzoyl)-4'-piperidylidenyl]propionate glycol (11). This procedure starting with 8 was the same as that used to convert 7 to 10, except that evap of the solvent after drying with Na_2SO_4 afforded a colorless oil which showed 2 spots on silica TLC (1:1 EtoAc/hexane). Flash chrom on silica gel (1:1 EtoAc/hexane) yielded a white solid (83%); mp 123-124°C; IR(KBr): 3500, 3340, 2950, 1735 and 1655 cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.42 (s, 3H, alkyl CH_3), 1.74 (br, m, 4H, 2 CH_2 adjacent

to the glycol, 3.4 (br, m, 4H, 2 CH_2 adjacent to the N_2), 3.88 (s, 3H, methyl ester), 4.59 and 4.78 (two br s, 2H, 2 OH of the glycol): MS, m/z 397 (M^+), 338, 320, 294, 195 and 167.

Ethyl-2-[N-(pentafluorobenzoyl)-4'-piperidylidenyl]propionate glycol (12). The olefin, 9 (1.2 g, 3.18 mmol) was dissolved in t-butanol (27 mL) and ice (9 g), then acetone (3 mL) was added and the solution was cooled to 0°C. A solution of $KMnO_4$ (1.0 g, 6.36 mmol) and NaOH (254 mg, 6.36 mmol) in H_2O (35 mL) was cooled to 0°C and added slowly. A solution of 10% sodium bisulfite (30 mL) was added after 5 min. After stirring for another 5 min, the aqueous layer was extracted 4 times with $CHCl_3$, and the combined organic extracts were washed with H_2O and dried. Evap gave a slightly brown solid which was recryst from methanol/hexane to give white crystals (1.19 g, 91%); mp 125-126°C; IR (film): 3520, 3350, 2920, 1730, 1655 cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.34 (t, 3H, CH_3 of the ethyl ester), 1.42 (s, 3H, alkyl CH_3 overlapped with the CH_3 of the ester), 1.73 (br m, 4H, 2 CH_2 adjacent to the glycol), 3.31 (br m, 4H, 2 CH_2 adjacent to the N_2) 4.34 (q, 2H, CH_2 of the ethyl ester), 4.54 and 4.78 (two br s, 2H, 2 OH of the glycol, the 4.54 overlapped with the CH_2 of the ethyl ester); MS, m/z 411 (M^+), 338, 320, 294 and 167.

N-(Pentafluorobenzoyl)-4-piperidylidenylacetic acid glycol (13). (Method A). The ester glycol 10 (1.047 g, 2.7 mmol) was dissolved in methanol (15 mL), and aqueous NaOH (20.5 mL, 0.2 N) was added. After refluxing for 30 min and adjusting the pH to 3 (10% HCl), the solution was extracted 5 times with EtoAc and the combined organic extract was washed (water) and dried. Evap afforded a white solid that was a single spot on TLC (1:9 methanol/EtoAc). Recryst from methanol yielded colorless crystals (955 mg, 96%); mp 295-299°C; IR (KBr): 3400, 2920, 1720 and 1635 cm^{-1}; ^1H NMR (DMSO-d_6): δ 1.65 (br m, 4H, 2 CH_2 adjacent to the glycol), 3.28 (br m, 4H, 2 CH_2 adjacent to the N_2), 3.70 (s, 1H, methine proton), 4.13 and 4.33 (two br s, 2H, 2 OH of the glycol), and 5.50 (br s, 1H, COOH, disappeared by addition of D_2O); MS, m/z 369 (M^+), 306, 294, 195 and 167.

2-[N-(Pentafluorobenzoyl)-4'-piperidylidenyl]propionic acid glycol (14).

This procedure, starting with 11, was the same as that used to convert 10 to 13. The white solid was recryst from methanol/chloroform to yield

colorless crystals (88%); mp 174°C; IR(KBr): 3440, 2940, 1730 and 1650 cm $^{-1}$; ^{1}H NMR (DMSO-d_6): δ 1.24 (s, 3H, alkyl CH_3), 1.70 (br m, 4H, 2 CH_2 adjacent to the glycol), 3.41 (br, m, 4H, 2 CH_2 adjacent to the N_2), 4.36 and 4.58 (two br s, 2H, 2 OH of the glycol), 4.74 (br s, CO_2H); MS, m/z 338, 320, 306, 195 and 167. Anal. calcd for $C_{15}H_{14}F_5NO_5$: C, 47.01; H, 3.68; F, 24.79; N, 3.65. Found: C, 47.15; H, 3.95; F, 25.00; N, 3.58.

N-(Pentafluorobenzoyl)-4-piperidylidenylacetic acid glycol (13). (Method B). The ester 7 (1.2 g, 3.44 mmol) was dissolved in methanol (15 mL) and aqueous sodium hydroxide (26 mL, 0.2 N) was added. The reaction was heated under reflux for 2.5 hr, and then poured over EtoAc. The aqueous layer was acidified with 10% HCl and the dried $CHCl_3$ extract (Na_2SO_4) was evap to yield a white solid (1.07 g, 96%) that was one spot on TLC (9:1 EtoAc/methanol); mp 94-96°C; IR (film): 3500, 2850, 1705, 1655 and 1625 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 2.42 and 3.06 (two t, 4H, 2 CH_2 adjacent to the carboxylic acid), 3.40 and 3.83 (two t, 4H, 2 CH_2 adjacent to the N_2), 5.73 (br, s, 1H, vinylic proton), and 9.83 (s, 1H, carboxyl proton); MS, m/z 335 (M^+), 294, 195, 167.

The olefinic acid (1.07 g, 3.19 mmol) was dissolved in dry THF (26 mL) and osmium tetroxide (973 mg, 3.8 mmol) and pyridine (4 mL) were added. After stirring at RT for 2 hr under N_2, a solution of 10% sodium bisulfite (30 mL) was added, followed by vigorous stirring for 1 hr, and then 10% HCl to pH 3. The combined ethyl acetate extractions (15 mL x 5) were washed with 10% sodium bisulfite, and H_2O, and dried. Evap gave 13 (783 mg, 66%), which had the same mp, mixed mp and spectral data of the dihydroxy acid prepared above.

N-[N'-(Pentafluorobenzoyl)-4-piperidylidenylacetyl]-glycine methyl ester glycol (15). The acid glycol, 13 (110 mg, 0.3 mmol) was suspended in chloroform (3 mL) and glycine methyl ester hydrochloride (188 mg, 1.5 mmol) and triethylamine (152 mg, 1.5 mmol) in $CHCl_3$ (1.5 mL) were added. 1-Hydroxybenzotriazole (40 mg, 0.3 mmol) was added and, after cooling the reaction to 0°C, dicyclohexylcarbodiimide (62 mg, 0.3 mmol) was added and the reaction was stirred at RT for 24 hr. Flash chrom (9:1 EtoAc/hexane) yielded a white solid (30 mg, 25%); mp 132°C; IR (KBr): 3400, 3320, 2920, 750, 1650 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 1.71 (br m, 4H, 2 CH_2 adjacent to the glycol), 3.36 (br m, 4H, 2 CH_2 adjacent to the N_2), 3.69 (s, 1H, methine proton), 3.72 (s, 3H, CH_3 ester), 4.02 (d, 2H, CH_2 adjacent to the

ester), 4.32 and 4.62 (br s, 2H, 2 OH of glycol), 7.44 (br t, 1H, NH); MS, m/z 440 (M^+), 423, 409, 315, 306, 195 and 167. Anal. calcd for $C_{17}H_{17}F_5N_2O_6$: C, 46.37; H, 3.89; N, 6.36. Found: C, 46.45; H, 4.06; N, 6.56.

m-(Pentafluorobenzyloxy)acetophenone (16a). (Method A). Pentafluorobenzyl bromide (2.62 g, 10 mmol), and m-hydroxyacetophenone (1.36 g, 10 mmol) were dissolved in 30 mL of CH_2Cl_2. Tetra-n-butylammonium sulfate (1.75 g, 5 mmol), and NaOH (2 g, 50 mmol) were dissolved in 20 mL of water. The organic phase was added all at once to the aqueous phase with vigorous stirring. After 3 hr of stirring at RT, the separated aqueous layer was extracted with 2 x 20 mL of CH_2Cl_2. The combined organic phase was washed with 5 x 15 mL of H_2O, and dried. Evap gave a white solid for flash chrom (1:9 EtoAc/hexane) to give a colorless solid which was recryst from hexane (2.9 g, 92%); mp 96-97°C; IR ($CHCl_3$): 3070, 2850, 1720, 1290, 1170 and 1030 cm^{-1}; ^1H NMR ($CDCl_3$):δ 2.60 (s, 3H, CH_3), 5.20 (t, 2H, J = 1.6 Hz, benzylic CH_2), and 7.47 (m, 4H, aromatic); exact mass calcd for $C_{15}H_9F_5O_2$: 316.0523, found: 316.0549.

Method B. Pentafluorobenzyl bromide (2.61 g, 10 mmol) and m-hydroxyacetophenone (1.36 g, 10 mmol) were dissolved in 30 mL of acetone and potassium carbonate (6.9 g, 50 mmol) was added. The reaction was stirred with reflux for 2 hr, then 20 mL of water was added and the product was extracted with EtoAc. The separated organic layer was washed with 20 mL of H_2O and dried. Evap and flash chrom gave the same product (2.73 g, 86%) as in Method A.

p-([N-Pentafluorobenzoyl,N-methyl]amino)acetophenone (16b). p-Aminoacetophenone (1.35 g, 10 mmol), suspended in 20 mL of dry benzene, was treated with 3 ml of triethylamine followed by a dropwise addition of pentafluorobenzoyl chloride (2.31 g, 10 mmol). After the reaction was stirred at RT for 30 min, 30 mL of EtoAc and 15 mL of 10% HCl were added. The organic layer wa washed with 20 mL of H_2O and dried. The product was one spot on TLC (1:5 EtoAc/hexane) and cryst from acetone as yellowish plates (1.77 g, 92%); mp 184°C; IR ($CDCl_3$): 3300, 3020, 2950, 1725, 1690 cm^{-1}; ^1H NMR ($CDCl_3$):δ 2.58 (s, 3H, CH_3), 6.43 (br s, H, NH) and 7.60 (two d, 4H, J = 8 Hz, aromatic). This product was pure enough for a methylation reaction

which was carried out by dissolving the amide (2.17 g, 6.6 mmol) in 60 mL of acetone, then K_2CO_3 (4.55 g, 33 mmol) and CH_3I (9.11 g, 66 mmol) were added. After the reaction mixture was stirred at RT under N_2 for 14 hr, 15 mL of H_2O and 30 mL of EtoAc were added. The organic layer was washed with H_2O and dried. Evap and flash chrom gave a white solid (2.10 g, 93%); mp 110°C; IR ($CDCl_3$): 3020, 2960, 1730, 1690 cm^{-1}; ^1H NMR ($CDCl_3$): δ 2.55 (s, 3H, CH_3), 3.50 (s, 3H, NCH_3), and 7.50 (two d, 4H, J = 8 Hz), exact mass calcd for $C_6H_{10}F_5NO_2$: 343.06244, found: 343.06318.

p-(Pentafluorobenzyloxy)acetophenone (16c). Pentafluorobenzyl bromide (1.044 g, 4 mmol), and p-hydroxyacetophenone (544 mg, 4 mmol) were dissolved in 25 mL of CH_2Cl_2. Tetra- n-butylammonium sulfate (680 mg, 2 mmol), and NaOH (0.4 gm, 10 mmol) were dissolved in 50 mL of H_2O. The reaction was carried out as described above to give, after flash column chrom (1:9 EtoAc/hexane), a colorless solid which was cryst from hexane (1.15 g, 91%); mp 92°C; IR ($CDCl_3$): 3055, 2960, 1725, 1255, 1170 cm^{-1}; ^1H NMR ($CDCl_3$): δ 2.51 (s, 3H, CH_3), 5.13 (t, 2H, J = 1.6 Hz, benzylic CH_2), 7.37 (two d, 4H, J = 9 Hz, aromatic); exact mass calcd for $C_{15}H_9F_5O_2$: 316.0523, found: 316.0532.

Methyl-[m-(pentafluorobenzyloxy)-E-3-methyl]cinnamate (17a) (plus side products 18a, 23 and 24) Method A. In a moisture free system, sodium hydride (360 mg, 15 mmol) was suspended in 100 mL of dry THF and methyldiethylphosphonoacetate (3.15 g, 15 mmol) was added. The reaction was stirred under N_2 at RT for 15 min, followed by addition of ketone 16a (3.16 g, 10 mmol). Stirring with reflux for 3 hr and then evap gave a yellowish oil for flash column chrom (1:9, EtoAc/hexane) to give four products: "E" isomer methyl ester 17a (2.65 g, 71%); "Z" isomer of methyl ester 18a (116 mg, 3%), "E" isomer ethyl ester 23 (355 mg, 9%); and "E" p-methoxy methyl ester 24 (174 mg, 4%). Compound 17a is a colorless oil; IR (film): 3055, 3020, 2955, 1725, 1640, 1295, 1215, 1135 and 1040 cm^{-1}; ^1H NMR ($CDCl_3$): δ 2.53 (d, 3H, J = 1.2 Hz, olefinic CH_3), 3.71 (s, 3H, methyl ester), 5.08 (t, 2H, J = 1.6 Hz, benzylic CH_2), 6.08 (q, 1H, J = 1.2 Hz, vinylic proton), and 7.15 (m, 4H, aromatic); exact mass calcd for $C_{18}H_{13}F_5O_3$: 372.0785, found: 372.0803. Compound 23 is colorless oil; IR (film): 3060, 3010, 2980, 1715, 1630, 1290, 1205, 1165 and 1035 cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.30 (t, 3H, J = 7 Hz, CH_3 of ester), 2.52 (d, 3H, olefinic CH_3), 4.12 (q, 2H, J = 7 Hz,

CH_2 of ester), 5.02 (t, 2H, J = 1.6 Hz, benzylic CH_2), 6.00 (q, 1H, vinylic proton), and 7.18 (m, 4H, aromatic); MS, m/z 386 (M^+). Compound 24 is a colorles oil; IR (film): 3060, 3010, 2950, 1720, 1635, 1290, 1210, 1165 and 1035 cm^{-1}; ^1H NMR ($CDCl_3$):δ 2.55 (d, 3H, J = 1.2 Hz, olefinic CH_3), 3.70 (s, 3H, methyl ester), 4.05 (t, 3H, J = 2 Hz, OCH_3), 5.08 (t, 2H, J = 1.6 Hz, benzylic CH_2), 6.08 (q, 1H, J = 1.2 Hz, vinylic proton), and 7.20 (m, 4H, aromatic); exact mass calcd for $C_{19}H_{16}F_4O_4$ 384.0984, found: 384.0992. Compound 18a is a colorless oil; IR (film): 3050, 3020, 2940, 1715, 1630, 1280, 1210, 1175 and 1030 cm^{-1}; ^1H NMR ($CDCl_3$):δ 2.17 (d, 3H, J = 1.2 Hz, olefinic CH_3), 3.53 (s, 3H, methyl ester), 5.10 (t, 2H, J = 1.6 Hz, benzylic CH_2), 5.88 (q, 1H, J = 1.2 Hz, vinylic proton) and 7.03 (m, 4H, aromatic); exact mass calcd for $C_{18}H_{13}F_5O_3$: 372.0785, found: 372.0758.

Method B. Electrophore 16a (1.83 g, 5.8 mmol) and methyl(triphenylphosphoranylidene)acetate 1.94 g, 5.8 mmol) were dissolved in dry xylene, followed by refluxing under N_2 for 34 hr. Evap (vac) and then flash chrom (1:9, EtoAc/hexane) gave starting material (630 mg), "E" isomer of methyl ester 17a (493 mg, 27%); and "Z" isomer of methyl ester 18a (314 mg, 17%). All the spectral data of 17a and 18a matched those prepared by Method A.

Ethyl-[m-(pentafluorobenzyloxy)-E-3-methyl]cinnamate (23). Sodium hydride (43 mg, 1.79 mmol) was suspended in 10 mL of dry THF, and diethylphosphate (276 mg, 1.79 mmol) was added. The reaction was stirred under N_2 for 15 min at RT, followed by addition of methyl ester 17a (556 mg, 1.5 mmol). The mixture stirred and refluxed for 1 hr, and then acidified with 10% HCl. EtoAc (20 ml) was added, and the organic layer was washed with H_2O and dried. Evap gave 23 as an oil (550 mg, 95%) with the same spectral data as above.

Methyl-([m-(p-methoxy-tetrafluorobenzyloxy)]-E-3-methyl)cinnamate (24).

Sodium (257 mg, 11.2 mmol) was dissolved in absolute methanol (10 mL) under N_2, and methyl ester 17a (830 mg, 2.2 mmol) was added. The reaction was stirred at RT for 3 hr, then evaporated to dryness with N_2 followed by flash chromatography (5:95 ethyl acetate/hexane), giving product (708 mg, 83%), and recovered starting ester (110 mg, 13%). The spectral data of compound 24 matched that prepared above.

Methyl-(p-[N-pentafluorobenzoyl,N-methylamino-E-3-methyl)cinnamate, 17b and Z isomer, 18b. The reaction was carried out as described above. A solution of ketone 16b (1.071 g, 3.12 mmol) in 5 mL of dry THF was added to a reaction mixture of NaH (90 mg, 3.75 mmol) and methyldiethylphosphonoacetate (787 mg, 3.75 mmol) in 10 mL of dry THF. After 16 hr of reflux and workup as before, followed by flash chrom, (3:7 EtoAc/hexane), two geometrical isomers, 17b and 18b (total 1.12 g, 86%, ratio by weight 6.7:1, respectively), were isolated. Compound 17b was a colorless oil; IR (film): 2950, 1720, 1675, 1635, and 1270 cm^{-1}; ^1H NMR ($CDCl_3$): δ 2.5 (d, 3H, J = 1.1 Hz, olefinic CH_3), 3.45 (s, 3H, NCH_3), 3.68 (s, 3H, methyl ester), 6.00 (d, 1H, J = 1.1 Hz, vinylic proton), and 7.20 (two d, 4H, J = 7 Hz, aromatic); MS, m/z 399 (M^+). Compound 18b is a colorless oil; IR (film): 2950, 1715, 1680, 1640, and 1260 cm^{-1}; ^1H NMR ($CDCl_3$): δ 2.13 (d, 3H, J = 1.11 Hz, olefinic CH_3), 3.42 (s, 3H, NCH_3), 3.66 (s, methyl ester), 5.80 (d, 1H, J = 1.1 Hz, vinylic proton), and 7.18 (two d, 4H, J = 7 Hz, aromatic).

Methyl-[p-(pentafluorobenzyloxy)-E-3-methyl]cinnamate, (17c) and Z isomer (18c). The reaction was carried out as described above. Sodium hydride (99 mg, 4.13 mmol) was suspended in 10 mL of dry THF and methyl-diethylphosphonoacetate (862 mg, 4.10 mmol) was added. The reaction was stirred under N_2 at RT for 15 min, followed by addition of ketone 16c (865 mg, 2.74 mmol). The reaction was refluxed for 7 hr, and workup as before gave two major products after flash chrom (1:9 ethyl acetate/hexane): "E" isomer of methyl ester 17c (748 mg, 75%), and "Z" isomer 18c (45 mg, 5%). Compound 17c is a colorless oil; IR (film): 3050, 3020, 2945, 1715, 1640, 1295, and 1040 cm^{-1}; ^1H NMR ($CDCl_3$): δ 2.53 (d, 3H, J = 1.2 Hz, olefinic CH_3), 3.68 (s, 3H, methyl ester), 5.03 (t, 2H, J = 1.6 Hz, benzylic CH_2), 6.01 (q, 1H, vinylic proton, and 7.03 (two d, 4H, J = 9 Hz, aromatic); MS, 372 (M^+). Compound 18c is a colorless oil; IR (film): 3050, 3015, 2935, 1710, 1625, 1210 and 1030 cm^{-1}; ^1H NMR ($CDCl_3$): δ 2.15 (d, 3H, J = 1.2 Hz, olefinic CH_3), 3.52 (s, 3H, methyl ester), 5.03 (t, 2H, 1.6 Hz, benzylic CH_3), 5.80 (q, 1H, J = 1.2 Hz, vinylic proton), and 7.08 (two d, 4H, Hz, aromatic).

m-(Pentafluorobenzyloxy)-E-3-methylcinnamoyl chloride (20). Carboxylic acid 19 (600 mg, 1.7 mmol) was suspended in 15 mL of dry benzene and thionyl chloride (3 mL, 41 mmol) was added. The reaction was heated at 90° for 20

h. Evap (vac), followed by evap of 2 x 10 mL of benzene, and drying (vac) overnight gave an oil (620 mg, 98%); IR (film): 3075, 3035, 2955,1770, 1655, 1285, 1205, 1130, 1055 and 945 cm^{-1}; ^1H NMR ($CDCl_3$): δ 2.51 (d, 3H, J = 1.2 Hz, olefinic CH_3), 5.16 (t, 2H, J = 1.6 Hz, benzylic CH_2), 6.41 (q, 1H, J = 1.2 Hz, vinylic proton), and 7.25 (m, 4H, aromatic); MS, m/z 376 $(M)^+$.

3'5'-bis-O-[(m-Pentafluorobenzyloxy)-E-3-methylcinnamoyl]-N3-methyl-thymidine (22). Thymidine derivative 21 (180 mg, 0.2 mmol) was dissolved in 5 mL of dry DMF. Potassium carbonate (135 mg, 1.0 mmol) and methyl iodide (277 mg, 2.0 mmol) were added. After stirring at RT under N_2 for 16 hr, 20 mL of EtoAc and 10 ml of water were added. The separated organic layer was washed with 3 x 10 mL of water and dried. Evap followed by flash chrom gave product (161 mg, 86%); mp 78-79°C; IR ($CDCl_3$): 3045, 2955, 1710, 1675, 1640, 1290, 1210, 1155, and cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.87 (br s, 3H, CH_3), 2.23 (m, 2H, CH_2), 2.57 (br s, 6H, two CH_3), 3.27 (s, 3H, NCH_3), 4.43 (m, 3H, CH_2 and CH), 5.01 (br s, 4H, two CH_2), 5.33 (m, 1H, CH), 6.07 (br s, 2H, two CH), 6.33 (t, 1H, J = 7 Hz, CH), 7.17 (m, 8H, two aromatic protons), and 7.27 (br s, 1H, CH overlapped with aromatic at 7.17). Anal. calcd for $C_{45}H_{34}F_{10}N_2O_9$: C, 57.70; H, 3.63; F, 20.30; N, 2.99. Found: C, 57.50; H, 3.57; F, 19.96; N, 3.09.

Isopropyl-[m-(pentafluorobenzyloxy)-E-3-methyl]cinnamate (25). Acid chloride 20 (125 mg, 0.24 mmol) was dissolved in 5 mL of isopropanol. After stirring at RT under N_2 for 15 min, evaporation gave an oil (124 mg, 95%) which was one spot on TLC (1:9 ethyl acetate/hexane); IR (film): 3068, 2980, 1715, 1635, 1290, 1210, 1170, 1110, and 1060 cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.28 (d, 6H, J = 6 Hz, two CH_3) 2.53 (d, 3H, J = 1.2 Hz, olefinic CH_3), 5.02 (sept., 1H, J = 6 Hz, CH), 5.08 (t, 2H, J = 1.6 Hz, benzylic CH_2 overlapped with CH ester at 5.02), 6.01 (q, 1H, J = 1.2 Hz, vinylic proton), and 7.18 (m, 4H aromatic); exact mass calcd for $C_{20}H_{17}F_5O_3$: 400.1098, found: 400.1125.

N-[m-(Pentafluorobenzyloxy)-E-3-methylcinnamyl]glycine methyl ester (26).

Methylglycinate hydrochoride (175 mg, 1.4 mmol) was suspended in 10 mL of acetonitrile and N-methylmorpholine (703 mg, 7 mmol) was added. After stirring at RT for 30 min, acid chloride 20 (524 mg, 1.4 mmol, dissolved in 5

mL of acetontrile) was added. The resulting solution was stirred for 15 min. EtoAc (25 mL) and 10 mL of 10% HCl were added. The separated organic layer was washed with 10 mL of 10% $NaHCO_3$, 10 mL of H_2O and dried. Evap gave crude product for flash chrom (3:7, ethyl acetate/hexane), yielding a colorless oil (357 mg, 60%); IR($CDCl_3$): 3440, 3325, 3080, 2965, 1750, 1670, 1635, 1520, 1215, 1140 and 1065 cm^{-1}; ^1H NMR ($CDCl_3$):δ 2.52 (d, 2H, J = 5.5 Hz, olefinic CH_3), 3.72 (s, 3H, methyl ester), 4.04 (d, 2H, J = 5.5 Hz, aliphatic CH_2), 5.07 (t, 1H, J = 5.5 Hz, NH), and 7.15 (m, 4H, aromatic); MS, m/z 340, 249, 188, 160, 132, 131, and 115.

Determination of 22 by GC-ECD. To a solution of 320 pg (3.4×10^{-13} mole) of 22 and 107 pg (3.4×10^{-13} mole) of 16c, the internal standard, in 100 ul of acetonitrile, was added 100 uL of an aqueous solution of 4.86 mM sodium periodate/0.126 mM potassium permanganate that had been adjusted to pH 8 with potassium carbonate. After 1 hr at room temperature, the solution was treated with 50 uL of toluene, vortexed, centrifuged, and 1 uL of the toluene layer was injected into a GC-ECD (Varian 3740 fitted with a 1905 on-column injector). GC column: HP-ultra, 5% phenylmethylsilicone, fused-silica capillary (15 m long, 0.5 uM film thickness, 0.32 mm id; Hewlett-Packard Company); injector: 50°C then programmed immediately after injection to 200 °C at 80° min^{-1}; detector 360°C; carrier gas: hydrogen at 110 mL min^{-1}.

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