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A New Procedure for the Labeling of Peptides and Amino Acids

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Abstract—Several stable, water soluble cyclohexa-2,4-dien-1-ones have been prepared and their photolytic coupling reactions in aqueous solution at 28 °C with various amino acids and dipeptides investigated. This procedure represents a new and high yielding method for the labeling of the terminal amino functionality of peptides and amino acids.

Introduction

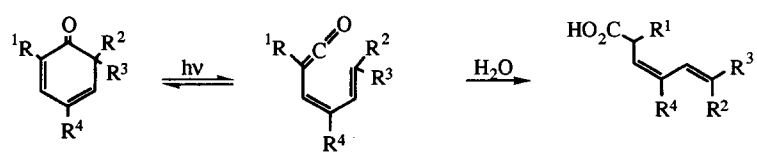
Some years ago we reported that upon irradiation with ultraviolet light, *ortho*-type cyclohexa-2,4-dien-1-ones (**1**) undergo smooth conversion to acids via nucleophilic trapping of the intermediate *cis*-ketene (**2**).[1] The mechanism[1–4] is depicted in Scheme I. Besides the capture of the ketene **2** with water, a number of other nucleophiles such as amines and alcohols have also been successfully employed, affording amides and esters in excellent yield.[1,2,5]

The photochemical sophistication of the reaction has been extensively studied by Quinkert and his colleagues and the process is well understood.[4] In addition, Quinkert has used the reaction as a key step in a number of elegant syntheses of complex natural products.[6] We felt that the full potential of this photochemical cleavage has not been utilized. One such application we felt plausible was the interception of the intermediate ketene **2** by amino acids and polypeptides affording derivatives of type **16/17**. It is envisaged that this type of procedure has potential for selective functionalization of complex polypeptides and proteins and the determination of nucleophilic functions on the external surface of a protein. A cyclohexadienone of type **1** would also serve to explore the surroundings of an active site when attached to the probe. The activation to a ketene affords a species which is more selective than the nitrenes or carbenes currently in use. The advantage over other methods is that the reaction can be carried out over a short time, in water at ambient temperature, all of which are highly desirable factors for peptide chemistry. This method makes use of the higher nucleophilicy of the terminal amine moiety of amino acids and peptides as compared to water and we report here our preliminary observations.

Results and Discussion

The requirements necessary to begin were that cyclohexa-2,4-dien-1-ones of type **1** must be water soluble at ambient temperature, stable and easily prepared. We began with **7a** and **b** in mind as depicted in Scheme II. Friedel–Crafts acylation of 2,6-dimethylphenol (**4**) afforded ketoester **5** in 96% yield. Reduction with alkaline zinc solution followed by re-esterification gave phenol **6** in moderate yield. Treatment of **6** with NaH followed by addition of chloromethyl methyl sulfide or chloromethyl phenyl sulfide yielded the dienones **7a** and **b** in moderate yields, (60–65%) along with recovered starting material (15%).

Both dienones **7a** and **b** were not, however, completely soluble in water at ambient temperature. Furthermore, unless careful attention was employed, desulfurization back to phenol **5** often occurred to varying amounts for **7a**. Such sensitivity is known.[5] In view of these facts, it was decided to further increase the water solubility, and stability, by converting **7b** to the corresponding sulfone **8**. This was accomplished as shown in Scheme II by employing MCPBA as the oxidant. The sulfone **8** proved to be stable but was still not readily soluble in water. However, hydrolysis of sulfone-ester **8** afforded quantitatively the sulfone-acid **9** which proved to be stable



1 **2** **3**

Scheme 1.

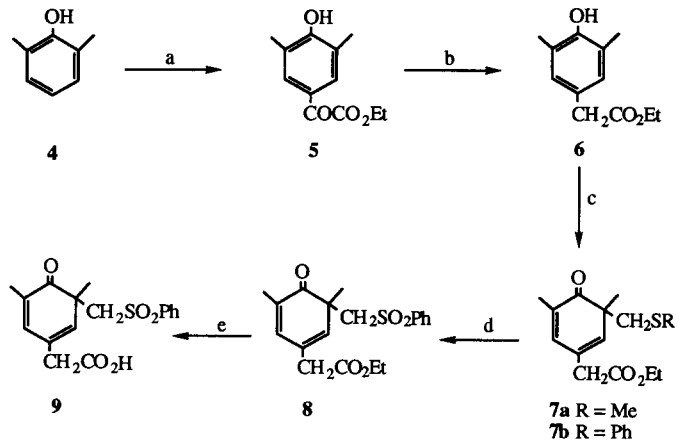
and readily water soluble at ambient temperature and was, therefore, used in the first photolytic studies.

Another synthetic approach to a water soluble cyclohexa-2,4-dien-1-one is highlighted in Scheme III. It was envisaged that incorporation of a phosphonate grouping into the dienone system would not only increase water solubility but also provide a handle in terms of monitoring the photolytic reactions and aid identification of the products by ^{31}P NMR. Also the ^{31}P spectrum could, with the aid of an internal standard, be used to quantify the number of ketenes that had reacted with a given protein or polypeptide.

Hydroxymethylation of 2,6-dimethylphenol (**4**) was accomplished with alkaline formaldehyde solution affording **10** in 75% yield. This material was quantitatively converted into chloride **11** with thionyl chloride which was further elaborated into the phosphonate **12** via an Arbuzov reaction.[7] *Ortho*-alkylation was not successful with the aforementioned NaH procedure owing to the formation of substantial quantities of inseparable *O*-alkylated product. This transformation was, however, possible with Corey–Kim reagent[8] affording the dienone **13** in excellent yield. Once again this type of dienone system was susceptible to fragmentation-desulfurization on prolonged standing. Thus it was immediately oxidized quantitatively to the stable and readily water soluble sulfone **14**.

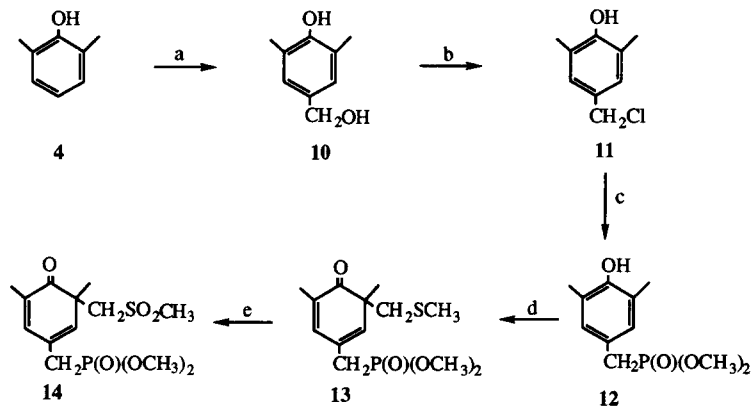
Photolysis of dienones 9 and 14 in the presence of amino acids

The results from photolysis of an aqueous solution of cyclohexa-2,4-dien-1-ones (**9** and **14**) containing added morpholine, amino acids or dipeptides are summarized in Table 1. All reactions were complete in less than 3 h and yielded in most cases the desired product in near quantitative yield. Although it is likely that the diene system is as shown[1–4] we cannot rule out the possibility that products derived from the *trans*-ketene are also formed. It was necessary to buffer the solutions to pH ≈ 10



(a) $ClCOCO_2Et$, $AlCl_3$, CS_2; (b) i) Zn, aq. NaOH ii) EtOH, H^+; (c) NaH, $RSCH_2Cl$
(d) MCPBA (2 equiv.), CH_2Cl_2, 25°C; (e) i) aq. NaOH ii) NH_4Cl.

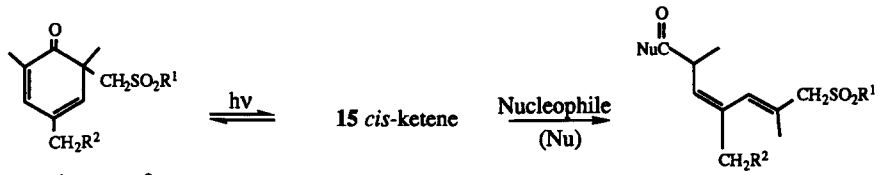
Scheme 2.



(a) aq. NaOH, CH_2O; (b) $SOCl_2$, 25°C; (c) $P(OCH_3)_3$; (d) NCS, $(CH_3)_2S$, NEt_3;
(e) MCPBA (2 equiv.), 25 °C.

Scheme 3.

Table 1. Photolytic reaction of aqueous **9** and **14** and various nucleophiles at 28 °C[a]



9 R^1 = Ph, R^2 = CO_2H
14 R^1 = CH_3, R^2 = $P(O)(OCH_3)_2$

16 R^1 = Ph, R^2 = CO_2H
17 R^1 = CH_3, R^2 = $P(O)(OCH_3)_2$

Dienone	Nucleophile (Equiv.[b])	Base (Equiv.[b])	Product (%)[c]	Selected ^{13}C and (^{31}P) NMR shifts
9	H_2O	—	**16a** (95)	180.36, 176.76
9	D_2O	—	**16a** (92)	—
9	Morpholine (10)	—	**16b** (93)	175.17, 173.16
9	Glycine (5)	K_2CO_3 (10)	**16c** (83)	174.43, 172.28, 171.15[e]
9	Gly-Glycine (5)	—	**16a** (90)	—
9	Gly-Glycine (5)	K_2CO_3 (10)	**16d** (89)	174.30, 172.45, 171.07, 170.08[e]
9	Gly-L-Leucine (2.7)	K_2CO_3 (10)	**16e** (68)	174.30, 173.80, 172.18, 169.56[e]
14	H_2O	—	**17a** (95)	177.53, (35.65)[f]
14	D_2O	—	**17a** (100)[d]	—
14	Glycine (5)	K_2CO_3 (10)	**17b** (83)	174.60, 171.40, (35.63)[f]
14	Gly-Glycine (5)	K_2CO_3 (10)	**17c** (84)	174.90, 171.68, 170.14, (35.63)[f]
14	Gly-L-Leucine (5)	K_2CO_3 (10)	**17d** (76)	174.89, 169.81, 169.61, (35.61)[f]

[a] See Experimental Section for full details. [b]Equivalents with respect to **9** or **14**. [c]Isolated yield. [d]NMR yield. [e]Recorded in acetone referenced to 29.6 ppm. [f] ^{31}P spectra were recorded in D_2O, all others recorded in $CDCl_3$.

(K_2CO_3) for the amino acids employed as the carboxylic acid resulting from water addition to the intermediate ketene was quantitatively formed at neutral pH. The reason for this difference in outcomes is easily reconciled when considering the structure of amino acids. At neutral pH, the nucleophilicity of the N-terminal is masked due to the amino moiety having a greater basicity with respect to the carboxylate anion. Hence capture of the ketene at pH ≈ 7 by water reflects the greater nucleophilicity of water with respect to the protonated amino group. Increasing the pH to approximately 10 with potassium carbonate liberates the amine portion of the amino acid and allows the amino group to react with it's normal nucleophilicity.

Concluding Remarks

The above reported results show the original hypothesis to be fully justified. When dealing with a protein or polypeptide an excess of the cyclic dienones would be employed. This is not a serious complication since any excess of dienone would eventually be converted into carboxylic acid and easily separated from the desired product of the reaction.

The 2-methyl group in **9** and **14** controls, sterically, the ease of hydration of the ketenes of type **2**. The methyl group may not be bulky enough and so the synthesis of reagents could begin with 2,6-diethylphenol or even with 2,6-diisopropylphenol. We have already referred above to quantification of the product by ^{31}P NMR spectroscopy. Another technique of importance for the determination of the number of ketenes which have reacted with a protein will be electron-spray mass spectroscopy.[11] Work is continuing and further results will be communicated in due course.

Experimental

Materials and instrumentation

Solvents were used as purchased or dried and purified by standard methods. Melting points were determined with a Kofler hot-stage melting point apparatus and are uncorrected. Boiling points are uncorrected. Infrared spectra were recorded on a Perkin-Elmer 881 spectrophotometer and referenced to a polystyrene standard while the UV/VIS spectra were measured with a Beckman Model DU-7 spectrometer. Proton (chemical shifts referenced to TMS at δ 0.00 in $CDCl_3$ unless otherwise stated), ^{13}C (referenced to $CDCl_3$ at δ 77.00) and ^{31}P (referenced to external 85% H_3PO_4) NMR spectra were measured at ambient temperature on a Varian XL-200 or a Gemini-200 spectrometer operating at 200, 50 and 81 MHz, respectively, using 5-mm tubes. ^1H and ^{13}C spectral data are presented as follows: multiplicity [*br* = broad, *s* = singlet, *d* = doublet, *t* = triplet, *q* = quartet, *p* = pentet, *m* = multiplet, *dd* = doublet of doublets, coupling constant (*J*), integration]. GC-MS analyses were performed on a Hewlett-Packard 5790A series gas chromatograph equipped with a quadrupole mass-selective detector. High resolution mass spectra were recorded on a VG Analytical 70S high resolution, double focusing, sectored (EB) mass spectrometer. High resolution FAB spectra were obtained with a 10 keV Xe beam at 2 mA (primary beam). Microanalyses were performed by Atlantic Microlab Inc., Norcross, GA. Reverse phase liquid chromatography was performed on reverse phase liquid chromatography column packing material obtained from Waters Associates; C18, 55-105 microns. All other chromatographic separations were performed with Silica Gel obtained from Baxter Scientific Products; 60Å, 230–400 Mesh.

General method for phototysis of cyclohexa-2,4-dien-1-ones (9 and 14)

Light from a Mercury Vapor Lamp (175 W) was employed to irradiate the dienone solutions contained in standard Pyrex glassware. To a solution of dienone, **9** or **14** (0.15 g) in H_2O (10 mL) at 28 °C was added the amine, amino acid or dipeptide, (See Table 1 for quantities). In some cases the solution was buffered with K_2CO_3, (See Table 1). The solution was then irradiated at a distance of 5 cm and the temperature kept below 30 °C by external cooling. The reactions were monitored by ^1H NMR and were all complete within 3 h. For **16a, b** and **17a** the H_2O was then removed *in vacuo* at 25 °C and the residue subjected to reverse phase liquid chromatography. For **16c–e** and **17b–d** the mixture was first acidified with 1 M citric acid to pH 3–4, followed by extraction with EtOAc (3 × 50 mL). Desiccation ($MgSO_4$) of the combined organic layers and removal of the solvent afforded crude **16c–e** and **17b–d** which were further purified by reverse phase liquid chromatography.

Ethyl-(3,5-dimethyl-4-hydroxy)benzoylformate (5). To a solution of 2,6-dimethylphenol (**4**) (30.7 g, 0.25 mol) and ethyloxalylchloride (28.0 g, 0.25 mol) in carbon disulfide (600 mL) at 0 °C was added aluminum chloride (66.0 g, 0.49 mol) portionwise over 30 min. Vigorous stirring was continued for 9 h at ambient temperature after which time the mixture was poured into ice-H_2O (1.2 L) and extracted with Et_2O (3 × 300 mL). The combined organic layers were then washed with H_2O (2 × 200 mL) and dried over magnesium sulfate. Removal of the volatiles *in vacuo* afforded crude **5** which was purified by recrystallization from $CHCl_3$:hexanes affording pure **5** as white needles; 52.3 g, 96%. Mp 113–114 °C; IR (KBr cm^{-1}) 3403, 2990, 1731, 1651, 1574, 1193, 1149, 905, 752, 702; ^1H NMR: δ 7.62 (*s*, 2H), 5.80 (*br s*, 1H), 4.38 (*q*, *J* = 7.0 Hz, 2H), 2.24 (*s*, 6H), 1.37 (*t*, 3H); ^{13}C NMR: δ 185.50, 164.52, 158.79, 131.32, 124.63, 123.74, 62.13, 15.81, 14.04; MS *m/z* (rel. int.) 222 (8), 149 (100), 91 (10), 77 (11).

Ethyl-1-(3,5-dimethyl-4-hydroxy)phenylacetate (6). Zinc powder (106.0 g, 1.6 mol) was added slowly to a mixture of **5** (6.0 g, 27.0 mmol) in alkaline solution (NaOH, 33.1 g; H_2O, 500 mL). The mixture was heated under reflux with vigorous stirring for 40 h, then cooled in an ice bath and acidified slowly to pH≈3 with conc HCl. Removal of the H_2O *in vacuo*, followed by extraction of the residue with EtOAc (3 × 300 mL), desiccation ($MgSO_4$), and removal of the volatiles gave the crude substituted phenylacetic acid as an off white solid. Recrystallization from Et_2O:hexanes afforded pure acid as a white crystalline solid (3.5 g, 70%). Mp 146–148 °C; IR (KBr cm^{-1}) 3444, 2997, 2968, 1681, 1290, 1196, 915, 896, 700, 630; ^1H NMR: δ 6.89 (*s*, 2H), 3.51 (*s*, 2H), 2.22 (*s*, 6H); ^{13}C NMR: δ 178.14, 151.69, 129.64, 124.93, 123.43, 40.02, 15.64. Esterification of the acid under normal conditions (EtOH, cat. H_2SO_4) afforded the acetate **6** (2.7 g, 67%) as a white crystalline solid after flash chromatography (Et_2O:hexanes, 1:4) along with recovered starting acid (25%). Mp 64–66 °C; IR (KBr cm^{-1}) 3464, 2986, 1707, 1471, 1439, 1303, 1194, 962, 874, 745; ^1H NMR: δ 6.87 (*s*, 2H), 4.86 (*s*, 1H), 4.14 (*q*, *J* = 7.2 Hz, 2H), 3.47 (*s*, 2H), 2.10 (*s*, 6H), 1.25 (*t*, *J* = 7.2 Hz, 3H); ^{13}C NMR: δ 172.47, 151.34, 129.25, 125.34, 123.35, 60.77, 40.45, 15.80, 14.11; MS *m/z* (rel. int.) 208 (26), 135 (100), 91 (8).

Synthesis of cyclohexa-2,4-dien-1-one (7a). To a solution of deoiled sodium hydride (96 mg, 2.4 mmol, 60% in mineral oil) in anhydrous benzene (20 mL) under argon was added **6** (0.415 g, 1.98 mmol) in anhydrous benzene (5 mL). After 1 h at ambient temperature, the mixture was chilled in an ice bath and chloromethyl methylsulfide (0.13 mL, 2.35 mmol) added via syringe. Stirring was continued for 4–5 h and then the solution was filtered, and the volatiles removed *in vacuo*. The residue was subjected to flash chromatography (EtOAc hexanes, 1:1) affording **7a** (0.33 g, 65%) as a pale yellow oil. IR (neat cm^{-1}) 2979, 2921, 1730, 1642, 1367, 1162, 977, 937, 769; ^1H NMR: δ 6.78–6.82 (*m*, 1H), 6.03–6.06 (*m*, 1H), 4.12 (*q*, *J* = 7.0 Hz, 2H), 3.18 (*s*, 2H), 2.78 (*dd*, *J* = 54.0 and 12.0 Hz, 2H), 2.00 (*s*, 3H), 1.86 (*s*, 3H), 1.22 (*t*, *J* = 7.0 Hz, 3H), 1.17 (*s*, 3H); ^{13}C NMR: δ 202.80, 170.72, 141.34, 140.64, 133.22, 126.22, 60.85, 51.16, 44.43, 40.56, 25.14, 17.58, 15.40, 14.09.

Synthesis of cyclohexa-2,4-dien-1-one (7b). The dienone **7b** was synthesized from **6** (9.35 g, 1.68 mmol) and chloromethyl phenylsulfide (0.25 mL, 1.87 mmol) in an identical manner to that described for **7a**, however, heating to 40 °C overnight was required. Purification by flash chromatography afforded pure **7b** (0.35 g, 63%) as a colorless oil. IR (neat cm^{-1}) 2980, 2925, 1729, 1642, 1581, 1478, 1253, 1176, 1025, 740, 690; ^1H NMR: δ 7.11–7.36 (*m*, 5H), 6.81–6.85 (*m*, 1H), 5.96–6.01 (*m*, 1H), 4.16 (*q*, *J* = 7.1 Hz, 2H), 3.25 (*dd*, *J* = 59.1 and 12.4 Hz, 2H), 3.12 (*s*, 2H), 1.85 (*s*, 3H), 1.27 (*t*, *J* = 7.1 Hz, 3H), 1.24 (*s*, 3H); ^{13}C NMR: δ 203.25, 170.66, 140.88, 140.55, 136.46, 133.32, 130.15, 128.64, 128.23, 126.26, 60.83, 50.76, 44.17, 40.43, 25.15, 15.35, 14.11; MS *m/z* (rel. int) 330 (6), 123 (100), 91 (8), 77 (9); Anal. Calcd for $C_{19}H_{22}O_3S$: C, 69.06; H, 6.71; S, 9.70. Found: C, 69.19; H, 7.74; S, 9.79.

Synthesis of cyclohexa-2,4-dien-1-one (8). To a solution of **7b** (1.1 g, 3.33 mmol) in dichloromethane (50 mL) at 0 °C was added MCPBA (1.78 g, 70% purity) dissolved in CH_2Cl_2 (15 mL). Stirring was continued for an additional 4 h after which time the organics were extracted with saturated aqueous $NaHCO_3$ solution (5 × 30 mL). Desiccation of the organic layer ($MgSO_4$) followed by removal of the volatiles *in vacuo* afforded crude **8** which was further purified by flash chromatography (EtOAc:hexanes, 1:9) to give **8** (1.05 g, 87%) as a white crystalline solid. Mp 144–145 °C ; IR (KBr cm^{-1}) 2974, 1731, 1639, 1299, 1177, 1153, 977, 784, 752, 686; ^1H NMR δ 7.73–7.80 (*m*, 2H), 7.40–7.62 (*m*, 3H), 6.74–6.80 (*m*, 1H), 6.18–6.22 (*m*, 1H), 4.17 (*q*, *J* = 7.0 Hz, 2H), 3.67 (*dd*, *J* = 168.0 and 14.0 Hz, 2H), 3.19 (*s*, 2H), 1.74 (*s*, 3H), 1.25 (*t*, *J* = 7.0 Hz, 3H), 1.14 (*s*, 3H); ^{13}C NMR: δ 200.35, 169.71, 140.55, 139.55, 139.43, 133.60, 132.73, 129.05, 128.33, 126.15, 64.35, 61.04, 48.33, 40.56, 27.07, 15.65, 14.16; MS *m/z* (rel. int.) 330 (6), 123 (100), 91 (8), 77 (9);

Anal. Calcd for $C_{19}H_{22}O_5S$: C, 62.95; H, 6.12; S, 8.85. Found: C, 62.14; H, 6.09; S, 8.79.

Synthesis of cyclohexa-2,4-dien-1-one (9). The ester **9** (0.55 g, 1.52 mmol) was dissolved in EtOH (20 mL) at 0 °C. To this mixture was added an aqueous solution of NaOH (15 mL, 15% w/w) in a dropwise manner and the temperature allowed to attain room temperature over 1 h. The mixture was then neutralized with NH_4Cl and concentrated *in vacuo* at 30 °C. The residue was then triturated with EtOAc (5 × 50 mL). The combined organic extracts were dried over magnesium sulfate and the volatiles removed *in vacuo* affording crude **9** as an off white solid. Further purification by flash chromatography (MeOH:$CHCl_3$, 1:19) gave the desired acid **9** (0.43 g, 90%) as a viscous white solid . IR (KBr cm^{-1}) 3400–2400, 1712, 1642, 1306, 1153, 983, 839, 686; ^1H NMR: δ 7.73–7.80 (*m*, 2H), 7.40–7.61 (*m*, 3H), 6.73–6.78 (*m*, 1H), 6.24–6.28 (*m*, 1H), 3.67 (*dd*, *J* = 165.4 and 14.0 Hz, 2H), 3.28 (*s*, 2H), 1.76 (*s*, 3H), 1.15 (*s*, 3H); ^{13}C NMR: δ 200.50, 176.02, 141.27, 138.87, 138.74, 133.80, 132.57, 129.19, 128.38, 126.84, 64.30, 47.68, 41.47, 27.09, 15.51; HRMS *m/z* Calcd for $C_{17}H_{18}O_5S$: $[M+H]^+$,335.09532. Found: 335.0954.

2,6-Dimethyl-4-hydroxymethylphenol (10). To a mixture of phenol **4** (6.1 g, 50.0 mmol) dissolved in alkaline solution (NaOH, 3 g / H_2O, 10 mL) at 5 °C was added formaldehyde solution (50 mL, 37% in H_2O). The mixture was then allowed to attain ambient temperature and stirring continued for 3 h after which time the mixture was neutralized with 1 M HCl at 0 °C and the solution extracted with EtOAc (4 × 200 mL). The combined organic layers were washed with saturated NaCl solution (2 × 100 mL), H_2O (2 × 100 mL) and dried over $MgSO_4$ and the volatiles removed *in vacuo*. Crude **10** was purified by flash chromatography (MeOH:$CHCl_3$, 1:19) affording pure phenol **10** (4.9 g, 64%) as a white crystalline solid. Mp 105–105.5 °C, Lit.[9] mp 104.5–105 °C; ^1H NMR: δ 6.98 (*s*, 2H), 4.6–4.9 (*br s*, 2H), 4.54 (*s*, 2H), 2.24 (*s*, 6H).

2,6-Dimethyl-4-chloromethylphenol (11). To a solution of **10** (7.6 g, 50.0 mmol) in anhydrous Et_2O (500 mL) under argon at 0 °C was added thionyl chloride (10.5 mL, 0.14 mol) dropwise over 10 min. The reaction mixture was stirred overnight after which time all volatiles were removed *in vacuo*. Recrystallization from hot benzene afforded **11** (8.45 g, 99%) as white needles. Mp 100–100.5 °C, Lit.[10] mp 100.5 °C; ^1H NMR: δ 7.00 (*s*, 2H), 4.52 (*s*, 1H), 4.50 (*s*, 2H), 2.23 (*s*, 6H).

Synthesis of cyclohexa-2,4-dien-1-one (12). Trimethylphosphite (6 mL, 0.05 mol) was added cautiously over 30 min to a solution of **11** (7.77 g, 45.8 mmol) in anhydrous benzene (250 mL) under argon at 0 °C. The mixture was heated under reflux for 15 h after which time the mixture was cooled, washed with brine (3 × 50 mL), dried ($MgSO_4$) and the solvent removed *in vacuo*. The residue was subjected to flash chromatography (EtOAc:hexane, 1:1) affording **12** (8.5 g, 76%) as a white crystalline solid. Mp 111–112 °C ; IR (KBr cm^{-1}) 3310, 2954, 2848, 1596, 1484, 1232, 1047, 1006, 884, 810, 722, 637; ^1H NMR: δ 6.80 (*s*, 2H), 6.0–6.3 (*br s*, 1H), 3.64 (*d*, *J* = 10.8 Hz, 6H), 2.98 (*d*, *J* = 21 Hz, 6H), 2.10 (*s*, 6H); MS *m/z* (rel. int.) 244 (26), 135 (100), 91 (10); Anal. Calcd for $C_{11}H_{17}O_4P$: C, 54.10; H, 7.02. Found: C, 54.25; H, 6.98. ^{31}P NMR ($CDCl_3$): δ 30.12.

Synthesis of cyclohexa-2,4-dien-1-one (13). Freshly distilled dimethylsulfide (3.3 mL, 45 mmol) was added dropwise to a mixture of *N*-chlorosuccinimide (5.8 g, 44.2 mmol) in dry CH_2Cl_2 (300 mL) under argon at –78 °C. After 2 h, phenol **12** (7.2 g, 29.5 mmol) dissolved in anhydrous CH_2Cl_2 (50 mL) was added dropwise so as to maintain the temperature below –70 °C. Stirring was continued for 3 h at this temperature after which time anhydrous Et_3N (6.3 mL, 14.3 mmol) was added and the mixture stirred for an additional 3 h at –78 °C. The mixture was then allowed to attain ambient temperature overnight. The mixture was then washed with cold 10% NaOH solution (2 × 100 mL), cold saturated NH_4Cl solution (2 × 100 mL) and brine (3 × 100 mL). Desiccation ($MgSO_4$) and removal of the volatiles *in vacuo* afforded crude **13** which was further purified by flash chromatography (Et_2O) affording **13** (9.0 g, 96%) as a pale yellow oil. IR (neat cm^{-1}) 2955, 2922, 2852, 1705, 1636, 1245, 1052, 1028, 977, 937, 809; ^1H NMR: δ 6.72–6.78 (*m*, 1H), 5.95–6.02 (*m*, 1H), 3.62 (*d*, *J* = 10.8 Hz, 6H), 2.71 (*dd*, *J* = 56 and 12.7 Hz, 2H), 2.62 (*d*, *J* = 21.6 Hz, 2H), 1.92 (*s*, 3H), 1.77 (*s*, 3H), 1.08 (*s*, 3H); MS *m/z* (rel. int.) 304 (48), 109 (20), 61 (100); ^{31}P NMR ($CDCl_3$): δ 28.63.

Synthesis of cyclohexa-2,4-dien-1-one (14). To a solution of **13** (7.14 g, 23.5 mmol) in CH_2Cl_2 (230 mL) at 0 °C was added MCPBA (14.8 g, 70% purity) dissolved in CH_2Cl_2 (100 mL). Stirring was continued for an additional 4 h after which time the organics were extracted with saturated aqueous $NaHCO_3$ solution (3 × 100 mL). Desiccation of the organic layer ($MgSO_4$) followed by removal of the volatiles *in vacuo* afforded crude **14** which was further purified by flash chromatography (MeOH:EtOAc, 1:19) to give **14** (6.0 g, 76%) as a white crystalline solid. Mp 125–127 °C; ^1H NMR: δ 6.78–6.84 (*m*, 1H), 6.17–6.22 (*m*, 1H), 3.65 (*d*, *J* = 10.8 Hz, 6H), 3.52 (*dd*, *J* = 157 and 14.3 Hz, 2H), 2.74 (*s*, 3H), 2.62 (*d*, *J* = 21.3 Hz, 2H), 1.84 (*s*, 3H), 1.15 (*s*, 3H); MS *m/z* (rel. int.) 336 (10), 257 (87), 229 (43), 147 (30), 119 (100); Anal. Calcd for $C_{13}H_{21}O_6PS$: C, 46.48; H, 6.29; S, 9.53. Found: C, 46.58; H, 6.42; S, 9.29. ^{31}P NMR: δ 34.86.

Product 16a (R^1 = Ph, R^2 = CO_2H, Nu = OH). Isolated as a waxy solid. IR (KBr cm^{-1}) 3400–2400, 1642, 1562, 1392, 1150, 951, 745, 685; ^1H NMR: δ 10.0–10.7 (*br s*, 1H), 7.84–7.96 (*m*, 2H), 7.48–7.70 (*m*, 3H), 5.56 (*s*, 1H), 5.45 (*d*, *J* = 9.8 Hz, 1H), 3.84 (*s*, 2H), 2.80–3.05 (*m*, 3H), 1.74 (*s*, 3H), 1.16 (*d*, *J* = 7 Hz, 3H); HRMS *m/z* Calcd for $C_{17}H_{21}O_6S$: $[M+H]^+$, 353.10589. Found: 353.1054.

Product 16b (R^1 = Ph, R^2 = CO_2H, Nu = C_4H_8NO). Isolated as a waxy solid. ^1H NMR: δ 10.0–10.5 (*br s*, 1H), 7.84–7.92 (*m*, 2H), 7.52–7.74 (*m*, 3H), 5.89 (*s*, 1H), 5.67 (*d*, *J* = 9.8 Hz, 1H), 3.77 (*s*, 2H), 3.40–3.75 (*m*, 9H), 3.07 (*s*, 2H), 1.80 (*s*, 3H), 1.19 (*d*, *J* = 7 Hz, 3H); HRMS *m/z*

Calcd for $C_{21}H_{27}NO_6S$: $[M+H]^+$, 422.16373. Found: 422.1643.

Product ***16c*** *(R^1 = Ph, R^2 = CO_2H, Nu = NH-CH_2-CO_2H).* Isolated as a waxy solid. ^1H NMR: δ 10.0–10.8 (*br s*, 1H), 7.90–8.00 (*m*, 2H), 7.60–7.78 (*m*, 3H), 7.10 (*s*, 1H); 5.74 (*s*, 1H), 5.50 (*d*, *J* = 9.9 Hz, 1H), 4.00 (*s*, 2H), 3.88–3.94 (*m*, 2H); 2.85–3.05 (*m*, 3H), 1.76 (*s*, 3H), 1.08 (*d*, *J* = 7.5 Hz, 3H); HRMS *m/z* Calcd for $C_{19}H_{24}NO_7S$: $[M+H]^+$, 410.12735. Found: 410.1257.

Product ***16d*** *(R^1 = Ph, R^2 = CO_2H, Nu = NH-CH_2-CO-NH-CH_2.CO_2H).* Isolated as a waxy solid. ^1H NMR: δ 10.2–10.6 (*br s*, 1H), 7.95–8.06 (*m*, 2H), 7.65–7.82 (*m*, 3H), 7.39 (*s*, 1H); 7.19 (*s*, 1H); 5.82 (*s*, 1H), 5.43 (*d*, *J* = 9.8 Hz, 1H), 4.06 (*s*, 2H), 3.86–4.04 (*m*, 4H); 2.88–3.12 (*m*, 3H), 1.80 (*s*, 3H), 1.14 (*d*, *J* = 7.5 Hz, 3H); HRMS *m/z* Calcd for $C_{21}H_{26}N_2O_8S$: $[M+H]^+$, 467.14881. Found: 467.1473.

Product ***16e*** *(R^1 = Ph, R^2 = CO_2H, Nu = NH-CH_2-CO-NH-CH(CO_2H)(Me_2CH-CH_2-).* Isolated as a waxy solid. ^1H NMR: δ 9.2–9.8 (*br s*, 1H), 7.85–7.92 (*m*, 2H), 7.50–7.72 (*m*, 3H), 7.20–7.40 (*br s*, 2H); 5.82 (*s*, 1H), 5.44 (*d*, *J* = 9.9 Hz, 1H), 4.40–4.54 (*m*, 1H), 3.70–4.15 (*m*, 4H); 2.85–3.18 (*m*, 3H), 1.60–1.80 (*m*, 5H), 1.12–1.38 (*m*, 4H); 0.80–1.00 (*m*, 6H); HRMS *m/z* Calcd for $C_{25}H_{35}N_2O_8S$: $[M+H]^+$, 523.21141. Found: 523.2119.

Product ***17a*** *(R^1 = Me, R^2 = P(O)$(OMe)_2$, Nu = OH).* Isolated as a waxy solid. ^1H NMR: δ 6.70–7.00 (*br s*, 1H), 6.02 (*s*, 1H), 5.49–5.56 (*m*, 1H), 3.68 (*d*, 6H), 3.10–3.20 (*m*, 1H); 2.94 (*s*, 3H), 2.63 (*d*, 2H), 1.82 (*s*, 3H), 1.18 (*d*, 3H); ^{31}P NMR: δ 35.65; HRMS *m/z* Calcd for $C_{13}H_{24}SO_7P$: $[M+H]^+$, 355.09804. Found: 355.0970.

Product ***17b*** *(R^1 = Me, R^2 = P(O)$(OMe)_2$, Nu = NH-CH_2-CO_2H).* Isolated as a waxy solid. ^1H NMR: δ 7.25–7.50 (*br s*, 1H); 6.90–7.00 (*br s*, 1H), 6.13 (*s*, 1H), 5.49–5.58 (*m*, 1H), 3.70–3.90 (*m*, 10H), 3.10–3.20 (*m*, 1H); 2.97 (*s*, 3H), 2.60–2.75 (*m*, 2H), 1.82 (*s*, 3H), 1.23 (d, 3H); ^{31}P NMR: δ 35.63; HRMS *m/z* Calcd for $C_{15}H_{26}NO_8PS$: $[M+H]^+$, 412.1195. Found: 412.1195.

Product ***17c*** *(R^1 = Me, R^2 = P(O)$(OMe)_2$, Nu = NH-CH_2-CO-NH-CH_2-CO_2H).* Isolated as a waxy solid. ^1H NMR: δ 7.15–7.30 (*br s*, 2H); 6.80–7.10 (*br s*, 1H), 6.13 (*s*, 1H), 5.48–5.58 (*m*, 1H), 3.65–3.95 (*m*, 12H), 3.10–3.22 (*m*, 1H); 2.98 (*s*, 3H), 2.60–2.78 (*m*, 2H), 1.82 (*s*, 3H), 1.20 (d, 3H); ^{31}P NMR: δ 35.65; HRMS *m/z* Calcd for $C_{17}H_{29}N_2O_9PS$: $(M+H)^+$, 469.14097. Found: 469.1415.

Product ***17d*** *(R^1 = Me, R^2 = P(O)$(OMe)_2$, Nu = NH-CH_2-CO-NHCH(CO_2H)(Me_2CHCH_2-).* Isolated as a waxy solid. ^{31}P NMR: δ 35.61; HRMS *m/z* Calcd for $C_{21}H_{37}N_2O_9PS$: $[M+H]^+$, 525.20357. Found: 525.2035.

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