A Novel Methodology for the Synthesis of Fumarates and Maleates

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Abstract: The stereoselectivity of both the Wittig and the Horner–Wadsworth–Emmons reactions allows for the synthesis of orthogonally protected fumarates and maleates, respectively, from α-keto esters. This methodology has been shown to be useful in the synthesis of a derivative of the pH sensitive *cis*-aconitic linker, important for prodrug approaches in drug delivery. We have incorporated this linker into a cholesterol-based PEGylated lipid, for its potential use in liposomal drug and gene delivery. The synthesized lipids have also been shown to be pH degradable using HPLC analyses.

Key words: Wittig, Horner–Wadsworth–Emmons (HWE), ozonolysis, *cis*-aconitic, PEGylated lipids

The use of cationic liposomes for the delivery of nucleic acids has shown great promise in recent years,[1] but has yet to provide a therapeutic solution. For efficient in vivo applications, which often require extended blood-circulation times,[2] liposome-nucleic acid (LD) systems require the attachment of a hydrophilic polymeric 'stealth' layer [commonly, polyethylene glycol (PEG)] to the liposome surface; usually through a PEG-functionalized lipid.[3] The down-side to these 'PEGylated' systems is that PEG inhibits the release of encapsulated nucleic acids at the site of therapeutic action.[4]

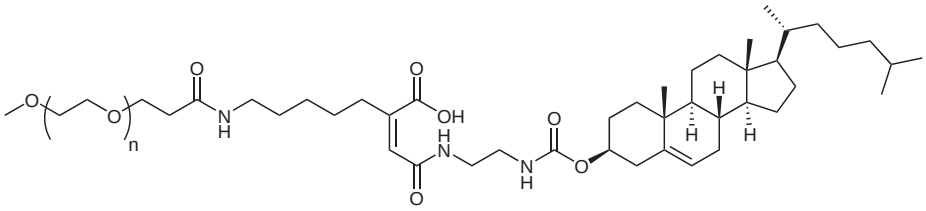
One solution to this problem has been to develop triggered release systems, wherein the PEG layer is naturally detached from the surface of the LD particles at the site of therapeutic action.[5] Researchers have made use of intrinsic cellular properties for this trigger effect, such as, the change in redox potentials,[6] varying enzyme concentrations[7] and changes in pH.[8]

LD particles usually enter cells by a mechanism known as endocytosis, a process that leads to their encapsulation within intracellular compartments known as endosomes.[9] These endosomes 'mature' with time and eventually fuse with lysosomes; a process whereby the pH of the compartment changes from pH 7.4 to slightly acidic pH 5.[10] Many of the triggerable systems currently being studied focus on this subtle pH change to facilitate the shedding of a 'stealth' layer. The main classes of bioconjugate linkers (usually incorporated between the lipid and PEG moieties) that have been shown to undergo acid-mediated hydrolysis include di-*ortho* esters,[11] hydrazones,[12] vinyl ethers[13] and the *cis*-aconitic acid linker.[14,15]

The *cis*-aconityl moiety has been shown to hydrolyse under mildly acidic conditions through the intramolecular formation of an unsaturated, cyclic anhydride[15] and release the potent *anti*-cancer drugs doxorubicin and daunomycin from HMPA polymers.[16]

In this communication, we present a methodology that allows for the facile synthesis of orthogonally protected maleate and fumarate functional groups. This methodology evolved from the earlier synthesis of a pH-sensitive sugar–lipid conjugate within our laboratories,[17] and from a study whereby we demonstrated the controlled and selective incorporation of the pH sensitive *cis*-aconitic linker into a PEGylated cholesterol-based lipid (**1a**–**c**, Figure 1).

Previous methods for introducing the *cis*-aconitic linker required simple nucleophilic reactions of appropriate amines with the commercially available *cis*-aconitic



1a, n = ~16 (PEG MW 750 g/mol)
1b, n = ~44 (PEG MW 2000 g/mol)
1c, n = ~112 (PEG MW 5000 g/mol)

Figure 1 The pH-sensitive cholesterol based PEGylated lipid (**1**)

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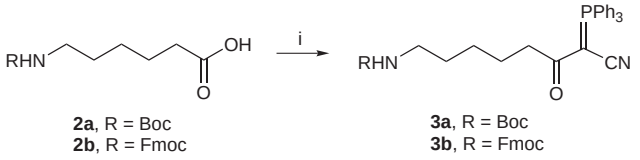
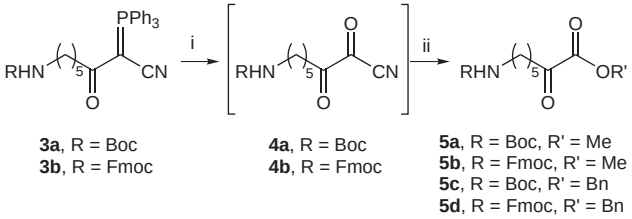
anhydride.[18] However, these conjugates are poorly characterized and *cis*-aconitic anhydride is known to undergo spontaneous *cis–trans* isomerization, leading to the undesired, non-pH-sensitive *trans* product.[19] Our previous synthesis of a glycolipid relied upon incorporating a derivative of the *cis*-aconityl linker through a de novo synthesis. One of the two key steps involved the treatment of an α-keto ester with a Horner–Wadsworth–Emmons (HWE) reagent, which generated an orthogonally protected maleate. However, this specific approach relied on a protecting group strategy not viable for general utility.[17]

We reasoned that treatment of α-keto esters with Wittig or HWE reagents would result in the selective formation of the respective fumarates and maleates. Furthermore, the incorporation of orthogonally protected acids would also allow for selective deprotections and subsequent regioselectivity in further synthetic manipulations.

Using the simple, 2-step route to α-keto esters developed by Wasserman,[20] we first coupled the commercially available N-protected 6-amino hexanoic acid (**2**) to triphenyl(phosphoranylidene)acetonitrile to form α-keto phosphorane **3**. The crude compounds were purified by silica gel flash chromatography to yield the desired products in 90% yields (Scheme 1).



Scheme 1 *Reagents and conditions*: i) *N*-Boc- or *N*-Fmoc-protected amino hexanoic acid **2** (1 equiv), triphenyl(phosphoranylidene)acetonitrile (1 equiv), EDCI (1.3 equiv), DMAP (0.1 equiv), CH_2Cl_2, 0 °C to r.t., 12 h.



Scheme 2 *Reagents and conditions*: i) α-keto phosphorane **3**, CH_2Cl_2, O_3, –78 °C, 20 min; ii) flushed mixture with dry N_2 before adding the relevant alcohol (1.2 equiv).

Table 1 α-Keto Ester Formation

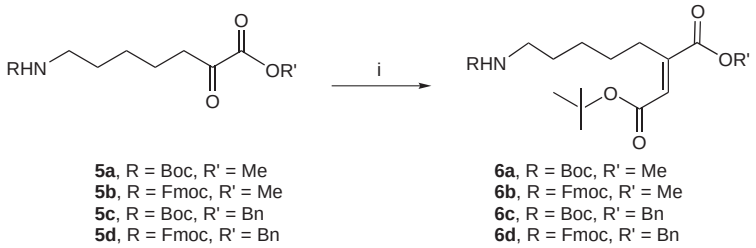
Entry	R	R′	Compound	Yield (%)
1	Boc	Me	**5a**	67
2	Fmoc	Me	**5b**	69
3	Boc	Bn	**5c**	58
4	Fmoc	Bn	**5d**	55

Second, the α-keto phosphoranes (**3a** and **3b**, Boc- and Fmoc-protected amines, respectively) were subjected to ozonolysis, forming the very electrophilic diketo nitrile (**4a**,**b**, not isolated). The nitrile intermediates were treated in situ with the required alcohol forming the α-keto esters **5a**–**d** (Scheme 2 and Table 1).[21] To introduce an orthogonal protection, two different esters were synthesized, a methyl ester and a benzyl ester, allowing for either acidic, basic or, in the case of R′ = Bn, hydrogenolysis deprotection protocols. Lower yields were obtained for the formation of the benzyl esters owing to the lower nucleophilicity of benzyl alcohol.

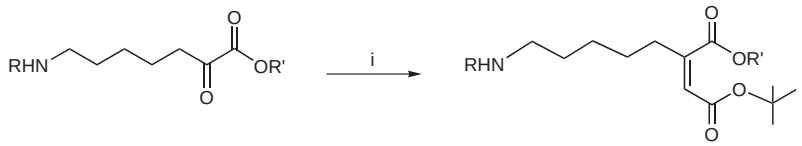
By treatment with classical Wittig and HWE reagents, the α-keto esters were selectively transformed into the respective fumarates and maleates. For the synthesis of the fumarates, the Wittig reagent *tert*-butoxycarbonylmethylene was added slowly to a solution of the α-keto ester **5a**–**d** in THF at 0 °C (Scheme 3).[22] ^{1}H NMR was used to determine the ratios of the *Z*- and *E*-products in the crude mixture, with the distinctive alkene protons showing at δ_H = 5.6 and 6.5 ppm, respectively. Simple NOE measurements confirmed the stereochemistry. The two isomers were easily separated using flash chromatography on silica gel, giving excellent yields and good selectivities for the *E*-products (**6a**–**d**, Table 2).

Table 2 Selective Synthesis of Orthogonally Protected Fumarates

Entry	R	R′	Compound	Yield (%)	*E:Z*
1	Boc	Me	**6a**	72	10:1
2	Fmoc	Me	**6b**	71	10:1
3	Boc	Bn	**6c**	76	8:1
4	Fmoc	Bn	**6d**	79	8:1



Scheme 3 *Reagents and conditions*: i) α-keto ester **5** (1 equiv), *tert*-butylcarbonylmethylene triphenylphosphorane (2 equiv), THF, 0 °C, 15 h.



5a, R = Boc, R' = Me
5b, R = Fmoc, R' = Me
5c, R = Boc, R' = Bn
5d, R = Fmoc, R' = Bn

7a, R = Boc, R' = Me
7b, R = Fmoc, R' = Me
7c, R = Boc, R' = Bn
7d, R = Fmoc, R' = Bn

Scheme 4 *Reagents and conditions*: i) α-keto ester **5** (1 equiv), *tert*-butyl-*P*,*P*′-dimethyl phosphonacetate (1 equiv), NaH (1 equiv), THF, 0 °C, 3 h.

The maleates were formed using the HWE reagent, *tert*-butyl-*P*,*P*′-dimethylphosphonoacetate. This reagent was first added dropwise to a solution of NaH in dry THF generating the anion, which was then added dropwise to the α-keto ester.[23] Again, ^{1}H NMR (and NOE measurements) was used to determine the ratios of the *E*- and *Z*-products from the crude mixture, with the two isomers easily separated by flash chromatography on silica gel. The reaction gave excellent selectivity for the maleates **7a**–**d**, in good yields (Table 3). The reduced yields for **7c** and **7d** can be reasonably explained by the steric interactions between the benzyl and *tert*-butyl groups. It is important to note that such steric interactions did not influence the *E*/*Z*-selectivites. Interestingly for these maleates the alkene proton signal could be observed as a triplet owing to small ^4J coupling (J = 1.4 Hz.).

Table 3 Selective Synthesis of Orthogonally Protected Maleates

Entry	R	R′	Compound	Yield (%)	*E*:*Z*
1	Boc	Me	**7a**	78	4:96
2	Fmoc	Me	**7b**	68	5:95
3	Boc	Bn	**7c**	50	4:96
4	Fmoc	Bn	**7d**	51	5:96

The methodology developed to provide the selective incorporation of the maleic moiety required for the formation of the *cis*-aconitic linker was next applied to the synthesis of three novel pH-triggerable, cholesterol-based PEG lipids (**1a**–**c**). In addition, the methodology developed to generate the fumaric moiety should find applications in the synthesis of non-pH-sensitive controls for our biological studies. To determine the effect of the PEG length on the hydrolysis rates of the linker, three PEG polymers were used: PEG 750 (n = 16), PEG 2000 (n = 44) and PEG 5000 (n = 112), to give their respective lipids **1a**, **1b** and **1c**.

Thus, the α-keto methyl ester **5a**, was treated with trimethyl phosphonoacetate under the HWE conditions to afford the corresponding dimethyl maleate **8** (confirmed by ^{1}H NMR and NOE measurements) in excellent yield (79%, Scheme 5). Maleate **8** was then subjected to regioselective hydrolysis of the less substituted methyl ester by using 1.1 equivalents of the LiOH. The regioselectivity of maleate **9**, was proved by ozonolysis followed by an oxidative work-up,[24] to afford α-keto methyl ester **5a** in 96% yield (Scheme 6).

The resultant acid **9** was activated with isobutyl chloroformate and then the in situ generated mixed anhydride was treated with cholesteryloxy-3-carbonyl-1,2-diaminoethane, affording cholesterol-derivative **10** in good yield (79%). The removal of the Boc group was achieved with a 1 M solution of hydrogen chloride in dry EtOAc.[25] The resultant amine hydrochloride **11** was reacted with the respective commercially available (Nektar Corporation, USA) NHS-activated PEG esters giving the PEGylated lipids **12a**–**c** (PEG 750, 2000 and 5000, respectively). Analytical HPLC was used to monitor the PEG coupling reactions. The final step required base-mediated hydrolysis of the methyl esters of **12a**–**c** to give the acid-labile PEGylated lipids **1a**–**c** as the regio- and stereopure maleates.

The rate of hydrolysis of the acid sensitive PEG lipids **1a**–**c** was evaluated at pH 5 and pH 7.4 in 0.1 M phosphate buffered saline (PBS) incubated at 37 °C. The percentage of degradation was determined by HPLC (Figure 2).

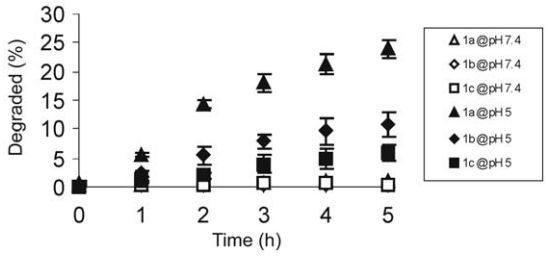
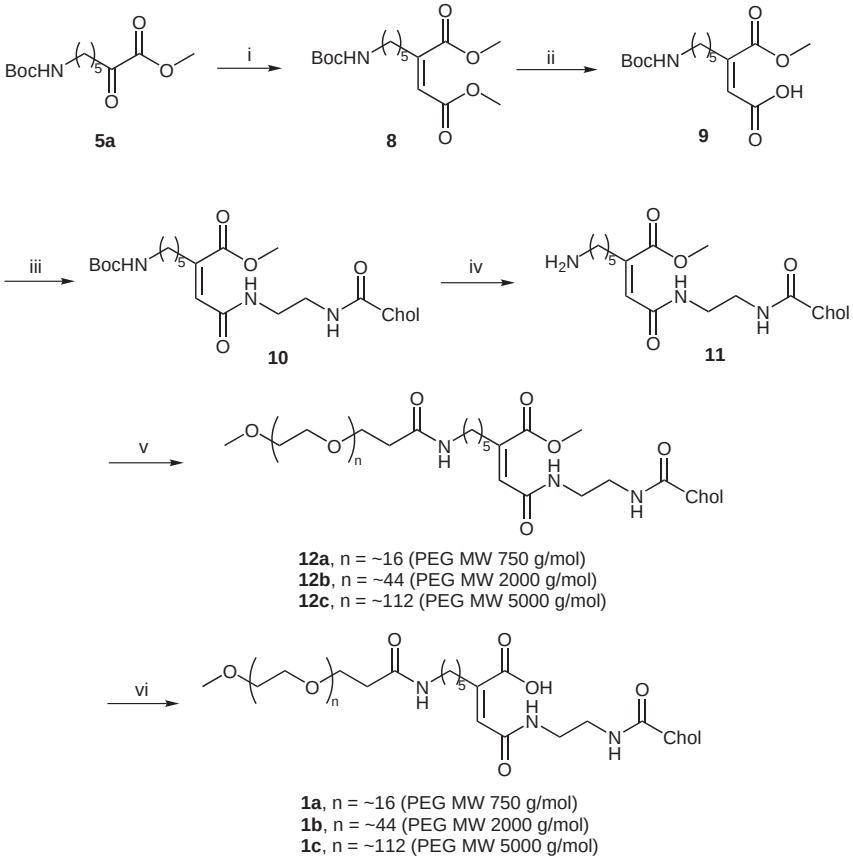


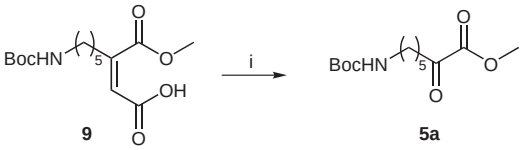
Figure 2 The degradation of the acid labile lipids **1a**–**c**

At physiological pH and temperature the lipids appear to be stable, with ≤1% hydrolysis over five hours. In a more acidic environment of pH 5, however, up to 25% of the lipids are hydrolyzed within the same time frame, thus demonstrating the pH sensitivity of the lipids. One observation that is clear from the data is that there was a significant difference in the hydrolysis rates of the linker depending on the length of the PEG polymer used. One possible explanation is that in solutions the lipids organize into entropically stabilized structures called micelles where the longer PEG lipids, arranged on the outside of the micelle, have sufficiently large 'hydrated' spheres that



Scheme 5 *Reagents and conditions*: i) trimethyl phosphonoacetate (1 equiv), α-keto ester **5a** (1.1 equiv), NaH (1 equiv), THF, 0 °C, 2 h (79%); ii) LiOH (1.1 equiv), THF–H_2O 3:1, 0 °C, 1 h (78%); iii) isobutyl chloroformate (1.1 equiv), NMM (2 equiv), after 10 min N′-cholesteryloxy-3-carbonyl-1,2-diaminoethane (1.1 equiv) was added, CH_2Cl_2, 0 °C to r.t., 12 h (79%); iv) 1 M HCl in EtOAc, 1 h (95%); v) PEG-NHS (0.9 equiv), NMM (1 equiv), CH_2Cl_2, r.t., 12 h, **12a** (69%), **12b** (67%), **12c** (58%); vi) 10% LiOH in THF–H_2O 3:1, 0 °C, 20 min, **1a** (79%), **1b** (72%), **1c** (71%); NOTE: Chol = cholesteryloxy.

limit the free exchange of protons to the acid-sensitive linkers. Although the lipids were shown to be acid labile the true test is to see how such molecules will behave in LD particles for nucleic acid delivery in in vitro cellular models.



Scheme 6 *Reagents and conditions*: i) maleate **9**, CH_2Cl_2, O_3, –78 °C, 20 min; ii) flushed mixture with dry N_2 before adding PPh_3 (4 equiv, 96%).

In conclusion, we have developed a facile, stereoselective synthesis for the formation of fumarates and maleates using Wittig and HWE reactions, respectively, in combination with Wasserman's α-keto ester procedure. We have presented the utility of this methodology for the development of steroidal lipids that are temporarily PEGylated through an engineered, pH-sensitive linker based on the *cis*-aconityl group.

References and Notes

(1) (a) Kosterelos, K.; Miller, A. D. *Chem. Soc. Rev*. **2005**, 970. (b) Fenske, D. B.; Cullis, P. R. *Methods Enzymol.* **2005**, 7.
(2) Gabizon, A.; Papahadjopoulos, D. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, 6949.
(3) Woodle, M. C.; Matthey, K. K.; Newman, M. S.; Hidayat, J. E.; Collins, L. R.; Redemann, C.; Martin, F. J.; Papahadjopolous, D. *Biochim. Biophys. Acta* **1992**, *1105*, 193.
(4) (a) Holland, J. W.; Hui, C.; Cullis, P. R.; Madden, T. D. *Biochemistry* **1996**, *35*, 2618. (b) Keller, M.; Harbottle, R. P.; Perouzel, E.; Colin, M.; Shah, L.; Rahim, A.; Vaysse, L.; Bergau, A.; Moritz, S.; Brahimi-Horn, C.; Coutelle, C.; Miller, A. D. *ChemBioChem* **2003**, *4*, 286.
(5) Guo, X.; Szoka, F. C. Jr. *Acc. Chem. Res.* **2003**, *36*, 335.
(6) Kirpotin, D.; Hong, K.; Mullah, N.; Papahadjopoulos, D.; Zalipsky, S. *FEBS Lett.* **1996**, *388*, 115.
(7) Pak, C. C.; Ali, S.; Janoff, A. S.; Meers, P. *Biochim. Biophys. Acta* **1998**, *1372*, 13.
(8) Kratz, F.; Beyer, U.; Schutte, M. T. *Crit. Rev. Ther. Drug Carrier Sys.* **1999**, 245.
(9) Zelphati, O.; Szoka, F. C. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 11493.
(10) Askon, A.; Cho, M. J. *J. Pharm. Sci.* **2002**, *91*, 903.
(11) (a) Guo, X.; Mackay, J. A.; Szoka, F. C. *Biophys. J.* **2003**, *84*, 1784. (b) Guo, X.; Szoka, F. C. *Bioconj. Chem.* **2001**, *12*, 291.

(12) (a) Hamann, P. R.; Hinman, L. M.; Hollander, I.; Beyer, C. F.; Lindh, D.; Holcomb, R.; Hallet, W.; Tsou, H. R.; Upeslacis, J.; Shochat, D.; Mountain, A.; Flowers, D. A.; Bernstein, I. *Bioconjugate Chem.* **2002**, *13*, 47. (b) Ulbrich, K.; Etrych, T.; Chytil, P.; Pechar, M.; Jelinkova, M.; Rihova, B. *Int. J. Pharm.* **2004**, *277*, 63. (c) Ulbrich, K.; Etrych, T.; Chytil, P.; Jelinkova, M.; Rihova, B. *J. Controlled Release* **2003**, *87*, 33.

(13) (a) Shin, J.; Shum, P.; Thompson, D. H. *J. Controlled Release* **2003**, *91*, 187. (b) Boomer, J. A.; Inerowicz, H. D.; Zhang, Z. Y.; Berstrand, N.; Edwards, K.; Kim, J. M.; Thompson, D. H. *Langmuir* **2003**, *19*, 6408.

(14) (a) Alshamkhani, A.; Duncan, R. *Int. J. Pharm.* **1995**, *122*, 107. (b) Reddy, J. A.; Low, P. S. *J. Controlled Release* **2000**, *64*, 2737. (c) Drummond, D. C.; Daleke, D. L. *Chem. Phys. Lipids* **1995**, *75*, 27.

(15) Bender, M. L. *J. Am. Chem. Soc.* **1957**, *79*, 1528.

(16) (a) Ulbrich, K.; Etrych, T.; Chytil, P.; Jelinkova, M.; Rihova, B. *J. Controlled Release* **2003**, 33. (b) Yoo, H. S.; Lee, E. A.; Park, T. G. *J. Controlled Release* **2002**, 17. (c) Drummond, D. C.; Daleke, D. L. *Chem. Phys. Lipids* **1995**, 27.

(17) Fletcher, S.; Jorgensen, M. R.; Miller, A. D. *Org Lett.* **2004**, 4245.

(18) Shen, W. C.; Ryser, H. J. P. *Biochem. Biophys. Res. Commun.* **1981**, *102*, 1048.

(19) Remenyi, J.; Balazs, B.; Toth, S.; Falus, A.; Toth, G.; Hudecz, F. *Biochem. Biophys. Res. Commun.* **2003**, *303*, 556.

(20) Wasserman, H. H.; Ho, W.-B. *J. Org. Chem.* **1994**, 4364.

(21) **General Procedure for the Formation of the α-Keto Esters.**
O_2 gas was bubbled through a solution of cyano keto phosphorane (2 mmol) in CH_2Cl_2 (25 mL) at r.t. for 10 min, cooled to –78 °C for 5 min. The solution then had O_3 bubbled through for 40 min at –78 °C until the color of the solution had changed to a murky green, via a bright yellow. Bubbling N_2 gas through the solution still at –78 °C for 10 min quenched the excess O_3, once the solution returned to bright yellow in color the alcohol (2.4 mmol) was added. The solution was then purged with N_2 and stirred at –78 °C for 2 h until the solution turned colorless. The crude product was purified using flash chromatography on silica gel to afford the α-keto ester as a clear oil.

(22) **Typical Procedure for the Formation of a Fumarate (6a).**
To a solution of the α-keto ester (0.25 mmol) in anhyd THF (6 mL) was added *tert*-butoxycarbonylmethylene triphenyl phosphorane (0.188 g, 0.5 mmol) dropwise (via syringe pump 2 mL/h) in anhyd THF (4 mL) at 0 °C under a nitrogen atmosphere. After 5 h the reaction was allowed to stir at r.t. for a further 10 h. The crude mixture was then concentrated in vacuo and purified using flash chromatography on silica gel to afford the desired fumarate.
IR (CH_2Cl_2 film): 3394, 2977, 2935, 1715, 1647, 1518, 1455, 1366, 1273, 1152, 974, 869 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ = 1.32–1.38 [2 H, m, $CH_2(CH_2)_2C$=C], 1.40–1.52 (4 H, m, 2 × CH_2), 1.43 (9 H, s, 3 × CH_3), 1.48 (9 H, s, 3 × CH_3), 2.72 (2 H, t, *J* = 7.6 Hz, CH_2C=C), 3.07–3.10 (2 H, m, CH_2NH), 3.77 (3 H, s, CH_3), 4.52 (1 H, br, NH), 6.65 (1 H, s, *trans*-alkene). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 26.73, 27.49 (2 × CH_2), 28.08, 28.48 [2 × $C(CH_3)_3$], 28.78 (CH_2), 29.72 (CH_2), 40.49 (CH_2NH), 52.35 (CH_3), 78.94, 81.34 [2 × $C(CH_3)_3$], 128.80 (CH=C), 145.80 (CH=C), 155.93 (CO_2NH), 164.95, 167.65 (2 × CO). MS (CI): *m/z* calcd for $C_{19}H_{34}N_1O_6$: 372.2386; found: 372.2382 [M^+ + H]; *m/z* (%) 389 (15) [M^+ + NH_4], 372 (100) [M^+ + H], 333 (76) [M^+ – $(CH_3)_3$ + NH_4], 272 (52) [M^+ – $(CH_3)_3CO_2$].

(23) **Typical Procedure for the Formation of Maleates.**
To a stirred suspension of NaH [0.01 g, 0.25 mmol (60% in mineral oil, w/w)] in anhyd THF (1 mL) at 0 °C and under a nitrogen atmosphere, was added *tert*-butyl-*P*,*P*-dimethylphosphonoacetate (0.05 mL, 0.25 mmol) in anhyd THF (1 mL) dropwise. After 30 min, the α-keto phosphorane (0.25 mmol) in THF (2 mL) was added dropwise over 1 h. After 3 h the excess NaH was neutralized with H_2O (0.1 mL) and the crude mixture concentrated in vacuo, azetroped with MeOH. The crude mixture was purified using flash chromatography on silica gel (hexane–EtOAc, 7:1) to afford the desired maleate. IR (neat): 3400, 2977, 2934, 2863, 1715, 1519, 1367, 1248, 1159, 1066, 1003, 781 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ = 1.32–1.38 [2 H, m, $CH_2(CH_2)C$=C], 1.40–1.52 (4 H, m, 2 × CH_2), 1.43 (9 H, s, 3 × CH_3), 1.48 (9 H, s, 3 × CH_3), 2.31 (2 H, t, *J* = 7.2 Hz, CH_2C=C), 3.07–3.10 (2 H, m, CH_2NH), 3.77 (3 H, s, CH_3), 4.52 (1 H, br, NH), 5.69 (1 H, t, *J* = 1.4 Hz, *cis*-alkene). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 25.98 (CH_2), 26.64 (CH_2), 27.91 [*t*-Bu $C(CH_3)_3$], 28.32 [Boc $C(CH_3)_3$], 29.68 (CH_2), 34.08 (CH_2C=C), 40.30 (CH_2NH), 52.00 (CH_3), 78.96 [Boc $C(CH_3)_3$], 81.33 [*t*-Bu $C(CH_3)_3$], 121.90 (CH=C), 147.83 (CH=C), 155.90 (CONH), 164.07 (CO), 169.25 (CO). MS (CI): *m/z* calcd for $C_{19}H_{37}N_2O_6$: 389.2651; found: 389.2645 [M^+ + NH_4^+]; *m/z* (%) = 389 (60) [M^+ + NH_4^+], 372 (80) [M^+ + H], 333 (65), 316 (35), 277 (100) and 216 (33).

(24) Diaz, M.; Branchadell, V.; Oliva, A.; Ortuno, R. M. *Tetrahedron* **1995**, *51*, 11841.

(25) Gibson, F. S.; Bergmeier, S. C.; Rapoport, H. *J. Org. Chem.* **1994**, *59*, 3216.