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A One-Pot Multistep Approach to α-Azido-phosphonate and Phosphonothioate Diesters: Key Intermediates in the Synthesis of Haptens for the Generation of Antibody Ligases

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Abstract—A four-step, one-pot synthesis of mixed α-azido-phosphonates and phosphonothioates **12a–d** is described. This chemistry has provided a facile route to haptens **6a–b** and **7** that have been employed for the elicitation of antibody ligases. Five hapten-specific antibodies have been identified as modest catalysts of a model peptide ligation reaction between thioester **1b** and thiol **2** to give the amide product **5**.

Chemical ligation is emerging as a valuable technique for the convergent synthesis of large proteins from unprotected peptide segments.[1] The acceleration of such chemoselective couplings of native or non-native peptide fragments is being explored with enzymes,[2] catalytic antibodies,[3–5] and self-replicating peptide sequences.[6]

Recently, the generation of peptides with native backbones has been achieved using an advanced chemical ligation process which combines a facile transthioesterification reaction with a spontaneous rearrangement to give an *N*-substituted amide precursor of the latent peptide bond.[7–9] We are seeking to exploit the known transesterification capabilities of antibodies[10,11] to facilitate a similar ligation approach. By targeting this type of cascade process, the desired product of antibody catalysis is a labile intermediate in the reaction sequence and thus its recognition should not inhibit turnover. The model system outlined in Scheme 1 has been devised to examine the feasibility of our strategy.[12]

The transesterification reaction between (thio)ester **1a–b** and thiol **2** to generate the intermediate **3** with concomitant release of **4a** or **b** represents the rate determining step in the formation of the ligation product **5**. Consequently, the mixed α-amidophosphonate diesters **6a–b** and **7** were designed as transition state analogues for the formation of thioester **3**.[13] The location of the glutaroyl linker in **6a–b** was chosen in an effort to promote recognition of the incoming nucleophile **2** in the antibody binding site and thus to direct its attack on the carbonyl center of **1a–b** in the presence of 55 M water.[14]

Phosphonic acid derivatives have been widely utilized in bioorganic chemistry, for example as mechanistic probes[15] and inhibitors[16] of peptidases. Methodology now exists whereby simple unsymmetrical phosphonate diesters and phosphonamidate esters can be obtained in 'one-pot' with good selectivity, using the tetrazole-catalyzed coupling of a phosphonyl dichloride with either two different alcohols,[17] or an alcohol and an amine.[18–19] However, reported procedures for the synthesis of highly functionalized mixed α-amino-phosphonates and phosphonamidates still employ a more laborious approach, involving the stepwise derivatization of polar mono- or bisphosphonic acids which themselves require initial purification by ion exchange or reverse-phase chromatography.[20] Consequently, the demand for a less circuitous route to generate complex mixed α-amido-phosphonate and phosphonothioates, such as haptens **6a–b** and **7**, served as a pivotal challenge within this project. Herein we describe a four-step, one-pot synthesis of mixed α-azido-phosphonate and phosphonothioate esters (Scheme 2), and demonstrate their smooth transformation into the respective α-amino and α-amido counterparts.

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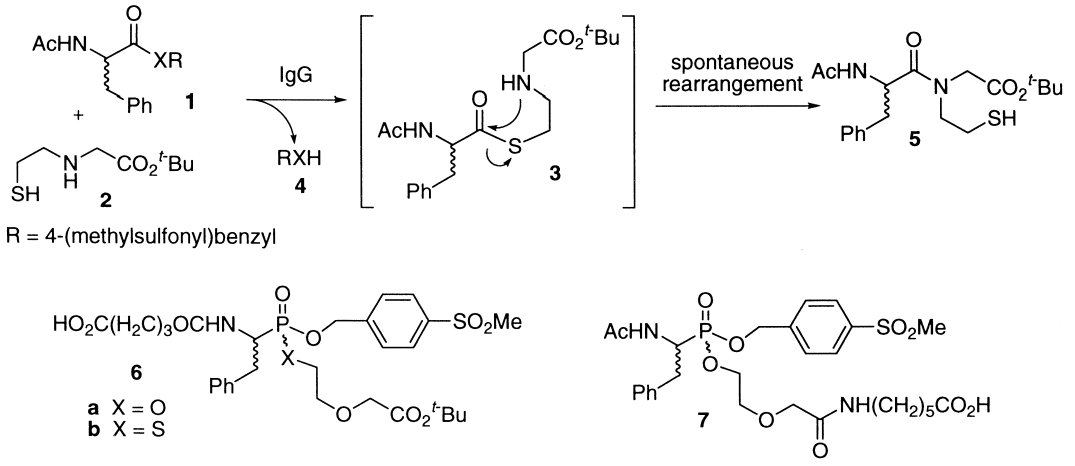
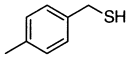
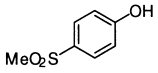
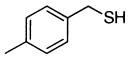
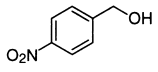
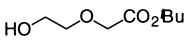
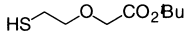


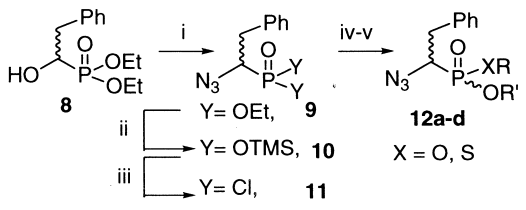
Scheme 1. Substrates **1a–b** and **2** and haptens **6a–b** and **7** were employed in a catalytic antibody approach to chemical ligation via transesterification.

Table 1. Four-step, one-pot approach to mixed α-azido-phosphonate and phosphonothioate diesters **12a–d**

Entry	RXH	R′OH	Product	Yield (%)[a]	^{31}P NMR[b] (ppm)
1			**12a**	24	55.8(s), 55.2(s)
2			**12b**	25	57.9(s), 57.1(s)
3	**4a**[23]	 **13a**[23]	**12c**	21	23.7(s), 23.3(s)
4	 **13b**[23]	**4a**[23]	**12d**	29	54.9(s), 54.5(s)

[a]Isolated yield after silica gel chromatography.
[b]Recorded on a Brüker AMX 400 NMR machine at 101 MHz; chemical shifts of diastereomers are reported downfield from an external reference (phosphoric acid).

Diethyl 1-hydroxy-2-phenethylphosphonate **8** was treated with hydrazoic acid under Mitsunobu conditions to generate the key phosphorus building block, azide **9**.[21] Then, in one reaction vessel azide **9** was first treated with excess bromotrimethylsilane[22] to generate the diester **10** which, in turn, was converted into phosphonyl dichloride **11** using oxalyl chloride with a catalytic amount of DMF.[23] The coupling to **11** was initiated, in the same pot, by the addition of either an alcohol or thiol



Scheme 2. Synthesis of α-azido-phosphonates and phosphonothioates. Reagents and conditions: (i) hydrazoic acid, DEAD, PPh_3, (ii) TMSBr, (iii) $(COCl)_2$, DMF (cat), CH_2Cl_2 (iv) iPr$_2$NEt, 1*H*-tetrazole (cat), CH_2Cl_2, RXH, (v) R′OH. Steps (ii)–(v) are carried out in 'one-pot'.

in the presence of 1*H*-tetrazole and DIPEA. After 15 min the second alcohol was added and the reaction mixture was stirred for a further 16 h. Silica gel chromatography furnished the mixed α-azido-phosphonothioates **12a,b,d** and phosphonates **12c** as diastereomeric mixtures in respectable yields (Table 1).[24] Easily accessible thiols and alcohols were employed to assess the utility of this approach (Table 1, entries 1 and 2) before the one-pot coupling procedure was performed using synthetic components **4a** and **13a/b** to generate hapten precursors **12c/d** (Table 1, entries 3 and 4), respectively.[25] This multistep process was far superior to existing stepwise methodology which, in our hands, furnished very poor yields of **12a–d** (0–10%). In addition, the polarity of the monophosphonic acid intermediates generated during the stepwise approach made their isolation troublesome.

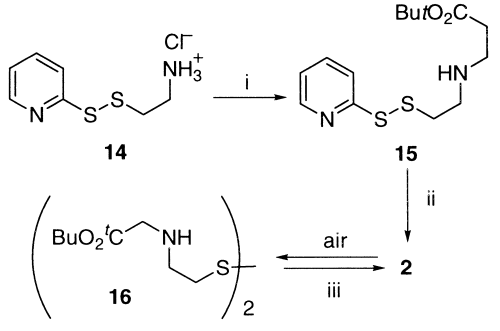
The α-azido-phosphonate **12c** was smoothly reduced to its corresponding α-amino derivative using 1,3-propanethiol[26] and subsequent acylation with glutaric anhydride afforded hapten **6a** in acceptable yield (45%). Hapten **7** was completed using an analogous reduction–

acetylation transformation of **12c** followed by attachment of a six-carbon linker.[27] The more robust azide moiety in **12d** was reduced with tin(II) chloride[28] and subsequent treatment with glutaric anhydride furnished hapten **6b** in 15% yield.

The (thio)ester substrates **1a–b** were formed by EDC mediated esterification of D,L-phenylalanine with **4a–b**, respectively.[29] The thiol substrate **2** was synthesized from *S*-(2-pyridylthio)cysteamine·HCl[30] **14** in two steps (Scheme 3). Alkylation of **14** with *t*-butyl bromoacetate in the presence of DIPEA yielded **15** (70%) which was then treated with *tris*-(2-carboxyethyl)phosphine·HCl (TCEP) to give substrate **2**. For ease of handling, thiol **2** was immediately converted into its corresponding disulfide **16** (80%) by exposure to air. A fresh stock of **2** was prepared immediately prior to kinetic assays by in situ treatment of **16** with an aqueous solution of TCEP.

Monoclonal antibodies with specific recognition of haptens **6a–b** or **7** were generated using established protocols.[31] These purified immunoglobulins were screened initially for their ability to accelerate the release of **4a–b** from esters **1a–b** in the presence of thiol **2** at pH 7.0 (100 mM MOPS, 2.5% DMSO).[32] Twelve antibodies capable of catalyzing the expulsion of **4b** were further investigated to ascertain the nature of their activity. By monitoring the formation of amide **5** (the spontaneous rearrangement product of **3**) during the reaction between **1b** and **2** in the presence of the antibodies, it was possible to distinguish thioesterase and transthioesterase mechanisms. This analysis revealed that five antibodies, three raised against **6a** and two raised against **6b**, catalyze the transesterification process. The remaining seven antibodies simply catalyze the hydrolysis of **1b**. Preliminary kinetic studies have revealed that the overall antibody-catalyzed rates (ca. 10^2) for the generation of **5** are encouraging but do not warrant in-depth investigation.

In conclusion, we have developed a facile four-step, one-pot coupling procedure for the synthesis of novel α-azido-phosphonate and phosphonothioate diesters. Representative examples **12c–d** have been exploited as intermediates for synthesis of haptens **6a–b** and **7**. Five of the antibodies generated against haptens **6a–b** catalyze the transthioesterification reaction between **1b** and **2**, but their catalyzed-rates do not merit extensive kinetic study. By incorporating the facile synthetic procedures reported in this paper, we are at present preparing new haptens in an effort to generate more powerful antibody ligases.



Scheme 3. Substrate synthesis. Reagents and conditions: (i) *t*-butyl bromoacetate, DIPEA, DMF, (ii) TCEP, EtOAc–H_2O, (iii) TCEP, H_2O.

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23. Removal of volatiles under a stream of argon furnished the crude intermediates **10** and **11**. ^{31}P NMR was used to monitor their formation: **10** (d 15.8 ppm) and **11** (δ 47.2 ppm).
24. Only minor amounts (< 5%) of the symmetrically substituted phosphonate diesters were observed.
25. Alcohol **4a** was prepared by reduction of 4-methylsulfonylbenzaldehyde with $NaBH_4$ in ethanol (95%). Alcohol **13a** was prepared in an overall yield of 14% by initial monoprotection of ethylene glycol (NaH, TBDMSCl), followed by coupling with *t*-butyl α-bromoacetate (50% aq NaOH, phase tranfer catalyst Bu_4NHSO_4) with removal of the TBDMS ether (TBAF) in the final step. Alcohol **13b** was prepared in three steps also (63% overall). The alcohol formed by the reaction of triphenylmethylmercaptan with 2-bromoethanol was etherified using *t*-butyl α-bromoacetate (vide supra). Finally, the *S*-trityl group was removed with $AgNO_3$. (Gupta, K. C.; Sharma, P.; Kumar, P.; Sathyanarayana, S. *Nucleic Acids Res*. **1991**, *19*, 3019).
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27. Hapten **7** was synthesized from **12c** in five steps (31% overall). After reduction of **12c** (propanethiol), acetylation was achieved using acetic anhydride (pyridine, DMAP). Removal of the *t*-butyl ester (TFA) allowed attachment of the protected linker *t*-butyl 6-aminohexanoate (EDC, HOBT, DIPEA). Deprotection of the resulting ester (TFA) furnished **7**. *t*-Butyl 6-aminohexanoate was synthesized in 74% yield from 6-aminohexanoic acid using benzyloxycarbonyl protection of the amine during *t*-butyl ester formation.
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31. KLH conjugates of **6a**–**b** and **7** were used to immunize 129Gix mice. Monoclonal antibodies were generated using hybridoma technology (Köhler, G.; Milstein, C. *Nature* **1975**, *256*, 495). Twenty-two monoclonal antibodies were elicited to KLH-**6a**, 25 monoclonal antibodies were elicited to KLH-**6b**, and 21 monoclonal antibodies were elicited to KLH-**7**.
32. In a typical screening assay, the disulfide **16** was mixed in equimolar amounts with TCEP in water for 5 min. To initiate the assay, this mixture was added to the aqueous buffer system [100 mM Bicine (pH 8.0), containing the esters or thioesters **1a**–**d** (500 μM) and 2.5% DMSO in the presence or absence of antibody (20 μM)] to give an initial thiol **2** concentration of 1 mM. The rates of reaction were measured by monitoring the rate of formation of **4a**–**b** (or **5**) by HPLC (adsorbosphere-HS column, acetonitrile:water (0.1% TFA) mobile phase, detection at 230 nm). The antibody-mediated rates (for < 5% of reaction, during which the progress curves were linear) were directly compared with the observed rate for the non-catalyzed reaction under identical assay conditions.