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Adducts between nucleophilic amino acids and hexahydrophthalic anhydride, a structure inducing both types I and IV allergy

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Abstract

Haptens causing type I allergy have been shown to predominantly form lysine adducts in the carrier protein, while many haptens giving rise to type IV allergy preferentially form adducts with cysteine residues. Hexahydrophthalic anhydride derivatives are strong sensitizers capable of inducing allergic rhinitis, asthma and urticaria (type I allergy) and allergic contact dermatitis (type IV allergy). The ability of hexahydrophthalic anhydride (HHPA) to form adducts with nucleophilic amino acids and a model peptide *in vitro* is presented. Adduct formation was monitored by high-performance liquid chromatography with ultraviolet light/vis detection (LC-UV/vis) and high-performance liquid chromatography with mass spectrometric detection (LC/MS). The characterization was obtained by nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS and MS/MS). It was found that HHPA formed adducts with N^{α}-acetylated lysine and cysteine and the non-acetylated α-amino group of proline and, to some extent, also with other nucleophilic amino acids. The adducts with lysine and proline were chemically stable. Addition of one HHPA to a model carrier peptide with all important nucleophilic amino acid residues showed N-terminal proline to be the major site of reaction. The addition of a second hapten gave a lysine adduct, but a minor cysteine adduct was also found. The cysteine–HHPA adducts were shown to be chemically unstable and participated in further reactions with lysine forming lysine–HHPA adducts. The results will be useful for understanding the formation of HHPA–protein adducts with the capability of being markers of exposure, and also to a deeper understanding of the chemical structures causing types I and IV allergy.

Keywords: *Adduct, allergy, amino acids, antigen, hapten, hexahydrophthalic anhydride*

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Introduction

Allergy is a common and increasing cause of human health problems today. Two of the most common types of allergy are type I allergy, with symptoms such as asthma, rhinitis, conjunctivitis and urticaria; and type IV allergy, with allergic contact dermatitis. Type IV allergy is induced by small reactive organic chemicals or metal salts (haptens) found in consumer goods, industrial chemicals and materials.

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Although type I allergy is mainly induced by proteins or peptides, some low molecular weight organic compounds can also induce this type of allergy.

To become immunogenic, haptens have to form adducts with carrier molecules. While haptens are chemically well defined, their metabolism in the tissue and their subsequent bonding to the carrier molecule has only been sporadically investigated. The original studies of Landsteiner and Jacobs (1936), Landsteiner and Chase (1941), Eisen et al. (1952) and Eisen (2001) indicated adduct formation between hapten and lysine in the carrier molecule, and these studies have greatly influenced the modern view on the formation of complete antigens. Several reports on adduct formation of haptens to lysine in model studies have been published (Liberato et al. 1981, Meschkat et al. 2001a,b). While the immunological literature is exclusively directed to lysine–hapten adducts, several haptens have molecular structures that, from a chemical point of view, are known as sulfhydryl reagents, i.e. they preferably form adducts with cysteine residues. We recently found that some type IV haptens, i.e. quinones, add exclusively to the cysteine moiety of a carrier peptide that also contained other nucleophilic amino acids (Ahlfors et al. 2003). Other strong sensitizers like 2,4-dinitrofluorobenzene form adducts with both lysine and cysteine residues, depending on the reaction conditions (Ahlfors, unpublished data). In our studies of type I haptens belonging to the organic acid anhydride group, we mainly found adducts to lysine and the *N*-terminal amino group (Lindh & Jönsson 1998, Kristiansson et al. 2002, 2003).

HHPA (**1**; Figure 1) and its derivatives are mainly used as monomers for manufacturing plastics. These anhydrides are haptens of particular interest since they are strong inducers of occupational rhinitis, conjunctivitis, urticaria and asthma by the formation of specific IgE antibodies (type I reaction) (Nielsen et al. 1994, Kanerva et al. 1999, Rosqvist et al. 2003) but also inducing T-cell-mediated allergic contact dermatitis (type IV reaction) (Kanerva et al. 1997). Both of these allergic reaction pathways are thought to be induced by the formation of a complete antigen through the formation of HHPA adducts to an endogenous protein, followed by degradation into a HHPA-modified oligopeptide. We performed our experiments at pH 5.5, representing the pH of the surface of the skin (Öhman & Vahlquist 1994), and at pH 7.4, representing physiological pH, to examine the whole pH gradient to which HHPA can be exposed.

Studies of the reactions between HHPA and different nucleophilic amino acids may be important from another point of view. It has been reported that HHPA forms adducts with haemoglobin (Hb) in humans (Jönsson et al. 1997). It has also been reported that HHPA binds to tissue in rat kidney (Lindh et al. 1999). Dannan et al. (1986) showed that cysteine adducts of acetylsalicylic anhydride rearranged to amides in the presence of an amine. This mechanism may also explain how the highly reactive HHPA forms adducts with the Hb and tissue in the kidneys. This led us to investigate the possibility that HHPA bound to *N*-acetyl-L-cysteine (NAcCys), could react further and could be transferred to N^{α}-acetyl-L-lysine (NAcLys).

HHPA is a reactive anhydride with a potential to react with several different amino acids. Therefore, HHPA is an interesting model compound for studies of allergenic chemical structures. The aim of this study was to investigate which amino acids form adducts with HHPA at skin pH and at physiological pH. In addition, we wanted to determine whether the initially formed adducts could take part in further reactions, resulting in other adducts.

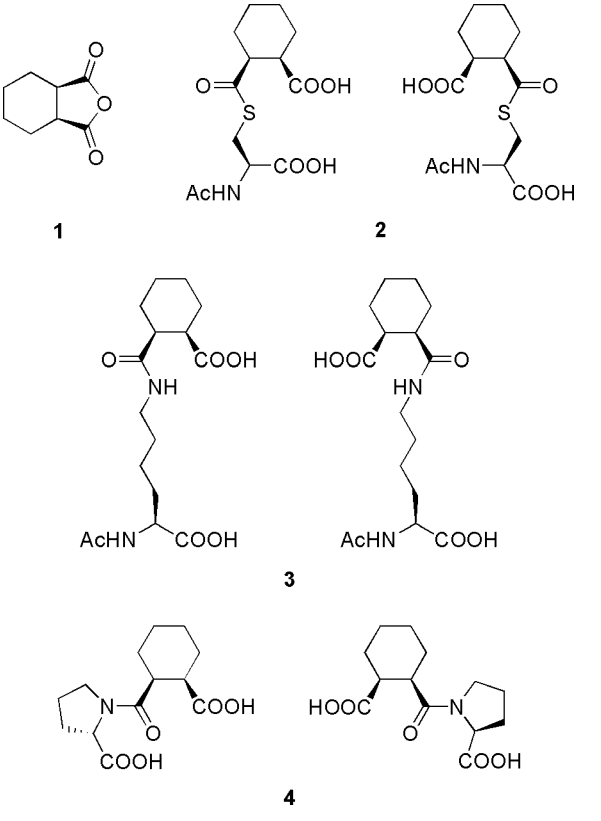

Figure 1. Chemical structures of *cis*-hexahydrophthalic anhydride (HHPA) **1**, *N*-acetyl-*S*-hexahydrophthaloyl-L-cysteine **2** (diastereomers), N^{α}-acetyl-N^{ε}-hexahydrophthaloyl-L-lysine **3**, and *N*-hexahydrophthaloyl-L-proline **4**.

Materials and methods

Chemicals and materials

Caution: HHPA is a highly allergenic chemical and should be handled with care. The chemicals used were as follows: cobalt (II) chloride, N^{α}-acetyl-L-arginine (NAcArg), N^{α}-acetyl-L-histidine (NAcHis), N^{α}-acetyl-L-lysine (NAcLys), *N*-acetyl-L-methionine (NAcMet), *N*-acetyl-DL-serine (NAcSer) *N*-acetyl-L-tyrosine (NAcTyr), N^{α}-acetyl-L-tryptophan (NAcTrp) (Aldrich, Milwaukee, WI, USA), H-Pro-His-Cys-Lys-Arg-Met-OH and sex pheromone inhibitor (iPD1) (Bachem, Bubendorf, Switzerland), sodium acetate (Fluka, Buchs, Switzerland), *N*-acetyl-L-cysteine (NAcCys), *cis*-hexahydrophthalic anhydride (HHPA 99%), *cis*-hexahydrophthalic acid (HHP acid), sodium dihydrogenphosphate monohydrate (Acros Organics, Geel, Belgium), acetic acid, disodium hydrogen phosphate dihydrate, formic acid, trifluoroacetic acid (TFA) (Merck, Darmstadt, Germany), acetonitrile, methanol (Lab-Scan, Dublin, Ireland), and L-proline, caesium iodide (Sigma Chemical Co., St Louis, MO, USA).

Medium nano electrospray (ES) capillaries were obtained from Protana Engineering A/S (Odense, Denmark). ZipTipC_{18} pipette tips were obtained from Millipore (Bedford, MA, USA).

Analytical LC

Analytical LC was performed with a Waters 600 pump (Waters Chromatography Division, Milford, MA, USA) equipped with a Rheodyne Model 7125 loop injector (20 μl) and a diode-array 1100 detector (LC-UV/vis; Hewlett Packard 1050; Palo Alto, CA, USA). The UV/vis spectra (190–900 nm) of every peak was recorded and evaluated. A linear gradient was applied from 5 to 80% acetonitrile in water with 0.1% TFA for 60 min, with a flow rate of 1.5 ml min^{-1}, and an EC Nucleosil 100-5C_{18} (5 μm, 4.6 mm i.d. × 150 mm) column (Macherey-Nagel, Düren, Germany), equipped with two 100-5C_{18} guard columns coupled in series (Phenomenex, Torrance, CA, USA), was used.

Preparative LC

Preparative LC was performed with a Waters 600E pump equipped with a Rheodyne Model 7125 loop injector (1.0 or 5.0 ml) and a Spectromonitor D (LDC/Milton Roy, Riviera Beach, FL, USA) UV detector working at 214 nm. Separations were performed on an Apex Prepsil C_8 (8 μm, 22 mm i.d. × 250 mm) column (Jones Chromatography, Lakewood, CA, USA). The products were separated using a linear gradient from 5 to 80% acetonitrile in water containing 0.1% TFA for 60 min, with a flow rate of 9.0 ml min^{-1}.

Mass spectrometric analysis of synthesized compounds

The compounds were dissolved in water containing 0.5% TFA and then purified using ZipTipC_{18} pipette tips. The compounds were eluted with an aqueous solution containing 50% methanol and 5% formic acid. Samples were analysed with hybrid quadrupole time-of-flight mass spectrometry (QqTOFMS; QSTAR; Applied Biosystems, Foster City, CA, USA) with a nanoelectrospray (nanoES) source. The mass spectrometer was calibrated using CsI (m/z 132.9054) and the pentapeptide sex pheromone inhibitor iPD1 (m/z 829.5398). Mass spectra were collected between m/z 50 and 2000. The ion spray voltage was set to 4500 V and the temperature of the auxiliary gas was 400°C.

NMR analysis of synthesized compounds

^{1}H-NMR spectra were recorded at 400 or 500 MHz and ^{13}C-NMR spectra at 100 MHz with Bruker DRX-400 or Bruker ARX-500 spectrometers (Bruker BioSpin, Rheinstetten, Germany). Acetic acid-d_4, methanol-d_4 and acetonitrile-d_3 were used as the solvent, and the solvent peaks (2.04, 20.0; 3.31, 49.15; 1.94 and 1.39 ppm) were used as internal standards. First-order chemical shifts and coupling constants were obtained from one-dimensional spectra, and the proton and carbon resonances were assigned from two-dimensional homonuclear correlation spectroscopy (COSY), ^{1}H-detected heteronuclear multiple-quantum coherence (HMQC), heteronuclear multiple-bond connectivity (HMBC) and total correlation spectroscopy (TOCSY) experiments.

Synthesis of N-acetyl-S-hexahydrophthaloyl-L-cysteine (NAcCys-HHPA) ***2***

NAcCys-HHPA was synthesized under anhydrous conditions with cobalt catalysis according to a modified method of Ahmad and Iqbal (1986). Anhydrous cobalt (II) chloride (42 mg) was added to a stirred solution of HHPA (74 mg) and NAcCys (64 mg) in 4 ml dry (3Å molecular sieves) acetonitrile at ambient temperature. When analytical LC showed complete reaction the solvent was evaporated, and the adducts **2** (Figure 1) were isolated (10 mg) by preparative LC. Data for product **2**: ^{1}H-NMR (400 MHz, CD_3CN) δ 6.74 (bd $J = 6.8$ Hz 1H NH), 4.55 (m 1H H-α), 3.41 (dd $J =$ 4.6, 14.0 Hz 1H H-β), 3.19 (m 1H H-β), 3.01 (m 1H H-1), 2.85 (m 1H H-2), 2.00–1.73 (m 4H H-3 H-6), 1.89 (s 3H $C\underline{H}_3$), 1.55–1.35 (m 4H H-4 H-5); ^{13}C-NMR (100 MHz, CD_3CN) δ 175.0, 171.9, 171.3, 55.3, 41.1, 26.7, 24.4, 22.9, 22.7. Product ion analysis by nanoES QqTOFMS of the singly charged ion at *m/z* 318.12 produced characteristic fragments at *m/z* 300.11 (loss of water), 272.09 (immonium ion of NAcCys-HHPA), 164.05 (NAcCys), 155.08 (HHPA) and 122.03 (Cys).

Synthesis of N^{α}-acetyl-N^{ε}-hexahydrophthaloyl-L-lysine (NAcLys-HHPA) ***3***

HHPA (463 mg) in 20 ml dry acetonitrile was added for 5 min while stirring at ambient temperature to a solution of NAcLys (188 mg) in 100 ml 0.1 M sodium phosphate buffer at pH 7.4. After 20 min of reaction the mixture was lyophilized, and the adduct was purified in portions by preparative LC. The yield from 200 mg lyophilized reaction mixture was 30 mg pure adduct **3** (Figure 1). Data for product **3**:^{1}H-NMR (400 MHz, CD_3OD) δ 4.33 (dd $J = 4.8$ and 8.9 Hz 1H H-α), 3.16 (t $J =$ 6.8 Hz 2H H-ε), 2.77 (m 1H H-2), 2.69 (m 1H H-1), 2.16 (m 1H H-3), 1.99 (s 3H CH_3CO), 1.94 (m 1H H-6), 1.83 (m 1H H-β), 1.75–1.69 (m 2H H-6 H-3), 1.67 (m 1H H-β), 1.64–1.56 (m 2H H-δ), 1.54–1.48 (m 2H H-γ), 1.47–1.36 (m 4H H-4 H-5); ^{13}C-NMR (100 MHz, CD_3OD) δ 177.92, 177.11, 175.95 C-1, 175.91 C-1, 173.5 $CH_3\underline{C}O$, 54.0 C-2, 44.7 C-1′, 44.6 C-1′, 44.0 C-2′, 43.9 C-2′, 40.04 C-6, 40.03 C-6, 32.4 C-3, 30.1 C-5, 28.4 C-3′, 28.3 C-3′, 27.9 C-6′, 27.8 C-6′, 25.0, 24.9, 24.8 C-5′, C-4′, 24.31 C-4, 24.30 C-4, 22.5 $\underline{C}H_3CO$. Product ion analysis by nanoES QqTOFMS of the singly charged ion at *m/z* 343.18 produced fragments at *m/z* 325.17 (loss of water), 237.16, 189.13 (NAcLys), 155.07 (HHPA) and 84.08 (immonium ion of lysine).

Synthesis of N-hexahydrophthaloyl-L-proline (Pro-HHPA) ***4***

HHPA (77 mg) in 1.87 ml dry acetonitrile was added during 5 min while stirring at ambient temperature to a solution of L-proline (57.5 mg) in 0.62 ml 0.1 M sodium phosphate buffer. After 2 h of reaction the mixture was lyophilized and the adducts (Figure 1) were purified in portions by preparative LC. Data for the two main *trans*-products **4** (first peak): ^{1}H-NMR (400 MHz, CD_3COOD) δ 4.48 (dd $J = 6.6$, 8.6 Hz 1H H-α), 3.53 (m 1H H-δ), 3.44 (m 1H H-δ), 2.93 (bt $J = 4.04$ Hz 2H H-1 H-2), 2.42 (m 1H H-β), 2.21 (m 1H H-β), 2.0 (m 4H H-γ (2H), H-3 H-6 1H each), 1.81 (m 2H H-3 H-6 1H each), 1.52 (m 2H H-4 H-5 1H each), 1.45 (m 2H H-4 H-5 1H each). Data for product 4 (second peak): ^{1}H-NMR (400 MHz, CD_3COOD) δ 4.53 (dd $J = 3.1$, 8.7 Hz 1H H-α), 3.80 (m 1H H-δ), 3.63 (m 1H H-δ), 3.22 (m 1H H-2), 2.67 (m 1H H-1), 2.24 (m 1H H-β), 2.1–2.0 (m 5H H-γ(2H), H-β H-3 H-6 1H each), 1.53 (m 2H H-4 H-5 1H each), 1.46 (m 2H H-4 H-5 1H each). Product ion

analysis by nanoES QqTOFMS of the singly charged product ion at m/z 270.13 resulted in fragments at m/z 252.12 (loss of water), 224.14 (immonium ion of Pro-HHPA), 155.07 (HHPA), 116.07 (Pro) and 70.06 (immonium ion of Pro).

Synthesis of HHPA-peptide adducts to proline ***5***

HHPA (10 mg; 64.8 μmol) in 1.0 ml dry acetonitrile was added slowly to a solution containing the hexapeptide (H-Pro-His-Cys-Lys-Arg-Met-OH; 50.0 mg; 64.8 μmol) in 25 ml 100 mM sodium acetate buffer while stirring under argon atmosphere. The pH of the solution was 5.5. After 3 h the crude reaction mixture was frozen in a CO_2 (s)/ethanol cooling bath, lyophilized and subjected to preparative LC. Analytical LC-UV/vis of the reaction mixture showed two main clearly separated peaks. A total of 2 mg pure adduct was isolated from the first peak, and 22 mg from the second one. Data for product **5** (second peak): ^{1}H-NMR shifts are shown in Table I and the structures in Figure 2. Product ion analysis by nanoES QqTOFMS of the doubly charged product ion at m/z 463.23 resulted in fragments at m/z 252.13 (154-modified $b_1^\star$ ion), 150.07 (y_1''), 306.18 (y_2''), 434.27 (y_3''), 537.28 (y_4'') and 674.34 (y_5'').

Adduct formation between HHPA and amino acids

The reactivity of HHPA to the nucleophilic L-amino acids arginine, cysteine, histidine, lysine, methionine, proline, serine, tryptophan and tyrosine was investigated at pHs 5.5 and 7.4. All amino acids were as their N^α-acetyl derivatives, except proline. Typical procedures at pHs 5.5 and 7.4 were as follows: solutions of the selected nucleophilic amino acid (10–200 mM) in either 100 mM sodium acetate (pH 5.5) or 100 mM sodium phosphate buffer (pH 7.4) were prepared and kept under an atmosphere of nitrogen or argon. Freshly prepared HHPA in dry acetonitrile (30 mg ml^{-1}) was added while stirring. Immediately after mixing, the reaction mixtures were analysed by LC and the analysis was repeated after 0.5, 1.5 and 3 h. To determine the relative stabilities of the HHPA adducts formed, LC analysis was repeated after 15, 24 and 48 h for the reactions that showed adduct formation. Several experiments were performed for each amino acid at pHs 5.5 and 7.4 and at several molar ratios from 1:1 to ten times excess of HHPA. The products were identified by NMR and nanoES QqTOFMS, and the adducts are shown in Figure 1.

Table I. ^{1}H-NMR data (500 MHz δ, ppm) for adduct 5 corresponding to peak b in Figure 5A.

Residue	H-α	H-β		H-γ		H-δ		Others
Pro	4.37	2.42	2.08	2.04		3.80	3.67	HHPA adduct[a]
His	4.52	3.35	3.26					8.51 H-2′ 7.31 H-5′
Cys	4.44	3.01	2.87					
Lys	4.35	1.88	1.75	1.49		1.69		2.93 H-ε
Arg	4.38	1.92	1.77	1.71		3.21		
Met	4.49	2.16	1.96	2.58	2.53			2.08 SCH_3

[a]Proton resonances from the HHPA part of the structure were in accordance with the ^{1}H-NMR data for the *trans*-ProHHPA adduct 4 (second peak).

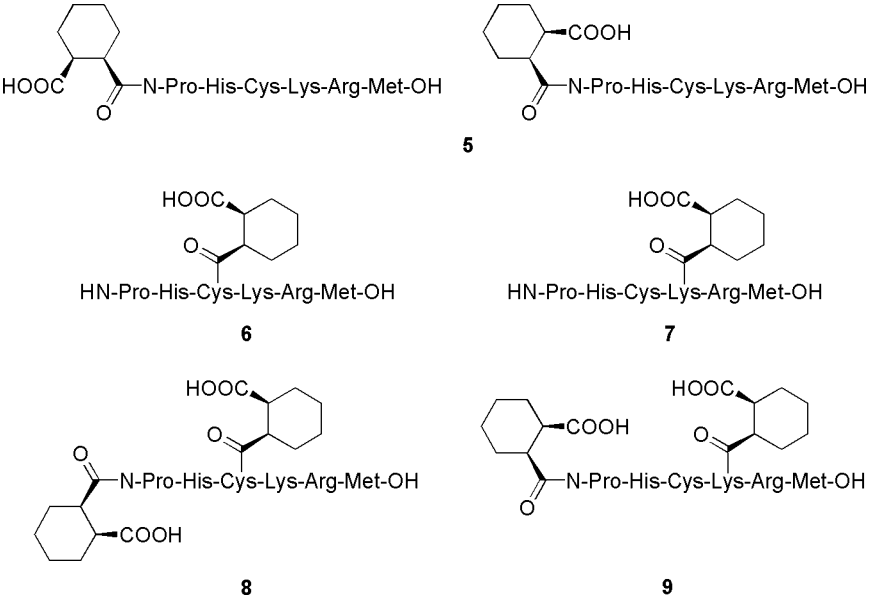

Figure 2. Chemical structures of *N*-hexahydrophthaloyl-Pro-His-Cys-Lys-Arg-Met-OH **5** (stereoisomers), the cysteine **6** and lysine **7** mono-adducts of the peptide, the peptide with HHPA at the proline and cysteine **8** and the peptide with HHPA at the proline and lysine **9**.

Adduct formation between HHPA and the hexapeptide

The reactivity of HHPA to the amino acids in the hexapeptide was investigated at pHs 5.5 and 7.4. A typical procedure was: HHPA (in portions of 10 µl, 64 mM in dry acetonitrile) was added, while stirring under nitrogen, to a solution of the peptide (630 µl, 1.31 mM) in 100 mM sodium acetate (pH 5.5) or 100 mM sodium phosphate (pH 7.4) buffer. Immediately after each addition of freshly prepared HHPA, the reaction mixtures were analysed by analytical LC-UV/vis as described for the study of adduct formation between HHPA and the amino acids. Several molar ratios of HHPA:peptide from 1:3, 1:1, 3:1 up to 100:1, were evaluated.

Analysis of HHPA-peptide adducts formed at pH 7.4 was also performed using LC-QqTOFMS with a turbo ion spray source coupled to an LC system (Hewlett Packard 1050). The LC parameters used were as above except that the flow rate was 0.8 ml min^{-1} and the TFA concentration 0.05%. Data-dependent experiments and product ion analyses were performed. The data-dependent experiments were carried out in cycles beginning with a TOF MS survey scan and followed by two product ion scans in which the highest abundant ion with a charge state of two or higher was selected. The MS parameters were: ion spray voltage 5 kV, temperature 420°C. Mass spectra were collected between m/z 50 and 2000.

Transfer of HHPA-adduct from N-acetyl-S-hexahydrophthaloyl-L-cysteine to N^{α}-acetyl-L-lysine

A 0.1 M phosphate buffer solution (800 µl; pH 7.4) containing 160 nmol NAcLys was prepared. A newly synthesized NAcCys-HHPA conjugate (**2** 1.6 µmol) was dissolved in dry acetonitrile (200 µl), added to the lysine solution, and incubated at 37°C. The

reaction between NAcCys-HHPA and NAcLys was monitored using a triple quadrupole mass spectrometer with electrospray ionization (API 3000; Applied Biosystems) coupled to an LC system from Perkin Elmer (200 series; Norwalk, CT, USA; LC/MS/MS). A reversed-phase C_{18} column with 5 μm particle size (4.6 mm i.d. × 50 mm; Aquasil, Thermo Hypersil-Keystone, Bellefonte, PA, USA) was used. The mobile phase consisted of water and methanol, both containing 0.5% acetic acid. A gradient elution was performed from 20 to 100% methanol during 0.5 min, isocratically at 100% methanol for 0.3 min and then returning to 20% methanol over 0.1 min in order to re-equilibrate the column for 0.6 min. The flow rate was 1 ml min^{-1} and 2 μl of the sample were injected. The mass spectrometric analysis was carried out with selected reaction monitoring in the positive-ion mode at transitions m/z: 343.2/236.8 (NAcLys-HHPA), 317.6/163.9 (NAcCys-HHPA), 189.0/128.9 (NAcLys), and 163.7/104.3 (NAcCys). The ion spray voltage was maintained at 4 kV and the temperature was set at 420°C. Before injection, the samples were diluted (20 μl sample to 480 μl 0.1 M phosphate buffer). The first sample was analysed 1 min after the addition of NAcCys-HHPA to the NAcLys-solution and from then on analysed every second minute.

Results

Adduct formation between HHPA and amino acids

Reactions between HHPA and the nucleophilic amino acids at pHs 5.5 and 7.4 were monitored by analytical LC-UV/vis. Adducts were observed to form in several cases (Table II).

LC-UV/vis analysis (Figure 3) showed a rapid reaction at both pHs 5.5 and 7.4 between NAcCys and HHPA. The identity of **2** was confirmed by comparison with the synthesized compound. The adduct formation was fast and reached a maximum after less than 30 min. The new products, probably corresponding to a diastereomeric pair, with retention times of 18.1 and 18.5 min had almost equal peak areas as determined by LC-UV/vis. However, it was not possible to separate the individual adducts by preparative LC, and ^{1}H-NMR spectroscopy in CD_3CN of the synthesized product did not show separated signals for the individual diastereomeric compounds. The adducts were not stable and were hydrolysed to free HHP acid and NAcCys at

Table II. Adduct formation between nucleophilic amino acids and HHPA at pHs 5.5 and 7.4 monitored by LC-UV/vis.

	pH 5.5	pH 7.4
NAcCys	+[a]	+
NAcMet	–[b]	–
NAcHis	–	+
NAcTrp	–	+
NAcSer	–	–
NAcTyr	–	+
NAcLys	–	+
NAcArg	–	–
Pro	+	+

[a]Formation of adducts.
[b]No formation of adducts.

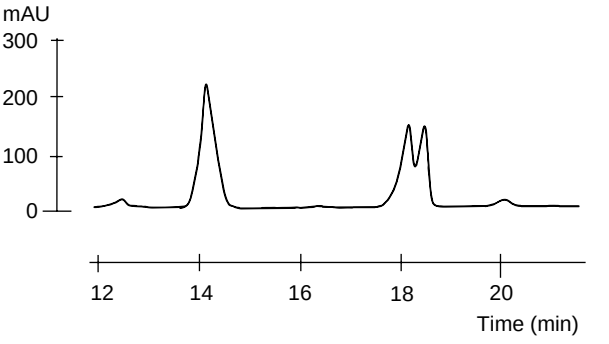

Figure 3. Typical analytical chromatogram of the reaction between HHPA and NAcCys recorded at 214 nm showing *N*-acetyl-*S*-hexahydrophthaloyl-L-cysteine **2** with retention times of 18.1 and 18.5 min, indicating two diastereomers, and the HHP acid at 14 min.

both pHs 5.5 and 7.4, but the hydrolysis at pH 5.5 was faster than at 7.4. However, after 24 h only small amounts of adduct could be detected at either of the pHs.

LC-UV/vis analysis indicated fast reactions between HHPA and NAcLys at pH 7.4. The identity of **3** (Figure 1) was confirmed by comparison with the synthesized compound. At pH 5.5 no adduct was detected. LC-UV/vis analysis of the reaction mixtures showed one broad double peak with a retention time of 14 min, with 10 s between the peak apexes. The reaction was fast and almost complete when using a HHPA:NAcLys molar ratio of 3:1. ^{1}H-NMR spectroscopy of the synthesized compound at 0, 20 and 50°C revealed different conformations (Figure 4). However, the ^{13}C-NMR spectra showed typical carbon shifts consistent with the presence of two diastereomers. The product was stable in buffer for several days.

Analysis of the reaction between HHPA and proline using LC-UV/vis showed the formation of two adducts, both at pHs 5.5 and 7.4. The retention times of these were 14.7 and 16.1 min and the identity of **4** was confirmed by comparison with the synthesized compounds. The reaction was rapid and almost complete when using a HHPA:Pro molar ratio of 3:1. ^{1}H-NMR spectroscopy showed the synthesized

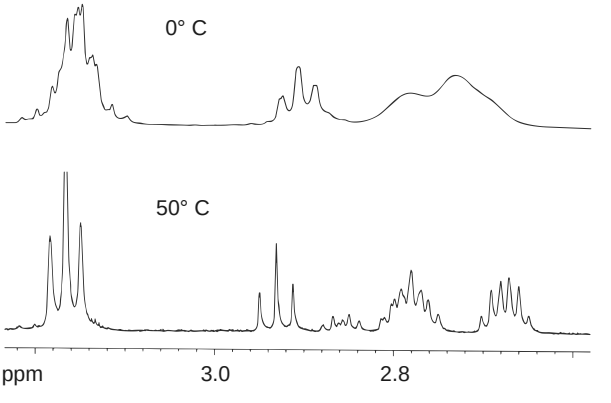

Figure 4. Part of the ^{1}H-NMR spectrum (400 MHz, CD_3OD) for N^{α}-acetyl-N^{ε}-hexahydrophthaloyl-L-lysine **3** at 0 and 50°C. The H-ε signal at 3.16 ppm shows typical conformational changes due to the barrier to rotation about the N^{ε}-amide bond. The H-1 signal at 2.69 ppm and the H-2 signal at 2.77 ppm from the cyclohexane ring also show conformational changes.

compounds to be diastereomers, and also revealed different *cis*/*trans* amide bond conformations for both isolated adducts that were comparable with published NMR data (Lee et al. 2001) for N-acetyl proline and proline containing peptides. The products were stable in buffer for several days but the *cis*/*trans* ratio changed slowly from 20/80 to 10/90 during 7 days in acetic acid at ambient temperature, according to ^{1}H-NMR spectroscopy of the adduct with 14.7 min retention time.

NAcTyr, NAcHis and NAcTrp also formed HHPA adducts at pH 7.4 (Table II). LC-UV/vis detection showed the formation of new products, but they were not isolated by preparative LC due to their unstable nature. However, it was possible to identify HHPA adducts of NAcTyr and NAcTrp with nanoES QqTOFMS (data not shown). The presence of NAcHis adducts could not be confirmed by nanoES QqTOFMS, possibly because of breakdown in the ion source.

Adduct formation between HHPA and the hexapeptide

Analytical LC-UV/vis showed immediate reaction between the model peptide and HHPA, at both pHs 5.5 and 7.4. The LC analysis of the reaction mixture at a molar ratio of 1:1 showed the formation of two new major products, having retention times of 16.4 and 18.5 min. NMR spectroscopy of the isolated substance with a retention time of 16.4 min indicated it to be substance **5** since two H-δ Pro signals at 3.7 ppm could be seen. NMR spectroscopy of the other product with a retention time of 18.5 min showed it to be a stereoisomer of **5** (Table I) since once again there was a characteristic shift change for the two H-δ Pro signals from 3.40 and 3.33 ppm in the peptide (Ahlfors et al. 2003), to 3.80 and 3.67 ppm in this adduct. These structures were also supported by comparison with the ^{1}H-NMR data of the two Pro-HHPA adducts **4**. The identities of the two stereoisomers of **5** were further confirmed by HMBC, HMQC and TOCSY experiments. However, we did not try to determine the absolute configuration of the isolated adducts.

The reactions between the hexapeptide and HHPA at pH 7.4 were also monitored with LC-QqTOFMS. The molar ratio between HHPA and peptide was 2:1. Product ion analysis of the doubly charged hexapeptide carrying one HHPA (*m*/*z* 463.24) resulted in four chromatographic peaks (Figure 5A), indicating at least four different isomers of the peptide adducts. The spectral data of the two major peaks consisted of identical y ion series: y_1'' (*m*/*z* 150.06) through y_5'' (*m*/*z* 674.33) that gave the sequence of the hexapeptide, and 154 Da-modified $b_1^{\star}$ ion (*m*/*z* 252.13) confirmed the position of the adduct to the N-terminal proline (Figure 5B). This was in accordance with the NMR data for the synthesized reference compounds. The two following peaks (c and d) contained only low-intensity mass spectral data. Nevertheless, peak c contained the unmodified ion y_3'' (*m*/*z* 434.26) and the 154 Da-modified ions $y_4''^{\star}$ (*m*/*z* 691.35) and $y_5''^{\star}$ (*m*/*z* 828.40), suggesting an HHPA adduct bound to the cysteine residue **6**. Peak d contained a very low abundant 154 Da-modified $y_3''^{\star}$ ion (*m*/*z* 588.34), as well as $y_4''^{\star}$ (*m*/*z* 691.35) and $y_5''^{\star}$ (*m*/*z* 828.41) suggesting a HHPA-adducted lysine **7**.

The LC-QqTOFMS data also indicated formation of adducts with two and three HHPA molecules bonded to the hexapeptide. Product ion analysis of the doubly charged ion at *m*/*z* 540.27 (hexapeptide carrying two HHPA) resulted in three chromatographic peaks of which the first being quite small (Figure 6A). Although, the signal was low, it was possible to detect the y ion series y_3'' (*m*/*z* 434.25) (unmodified),

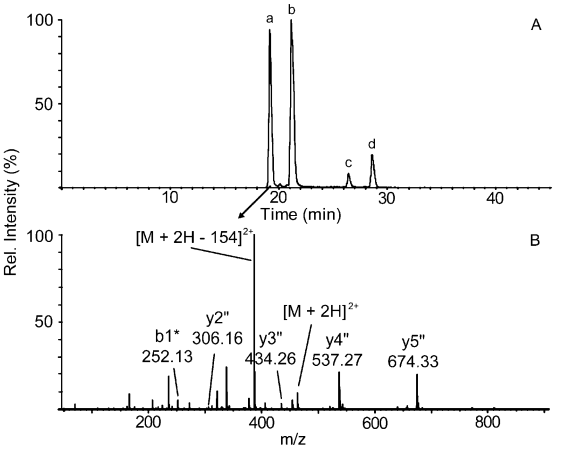

Figure 5. (A) Total-ion-current chromatogram obtained from product ion analysis of the doubly charged ion at m/z 463.23 of the hexapeptide H-Pro-His-Cys-Lys-Arg-Met-OH modified with one HHPA molecule in a phosphate buffer (pH 7.4), 3 h after synthesis. Analysis was performed with LC-QqTOFMS. (B) MS/MS mass spectrum of peak a contained the modified $b_1^{\star}$ ion at m/z 252.13 indicating the HHPA molecule bound to the N-terminal proline **5** Pro*-His-Cys-Lys-Arg-Met.

$y_4''^{\star}$ (m/z 691.33) and $y_5''^{\star}$ (m/z 828.38), both modified with 154 Da. Together with 154 Da-modified $b_1^{\star}$ (m/z 252.13) it was possible to determine the adducted sites to the N-terminal proline and most probably to the cysteine residue **8** for peak e (Figure 6B). The peaks f and g contained spectral data suggesting diastereomeric forms of the hexapeptide adducts, where one adduct was located to lysine and the other to N-terminal proline **9**. This was determined through the unmodified y ion series y_1'' (m/z 150.05) to y_2'' and 154 Da-modified $y_3''^{\star}$ (m/z 588.32), $y_4''^{\star}$ (m/z 691.32) and $y_5''^{\star}$ (m/z 674.32). The $b_1^{\star}$ (m/z 252.12) was also present. The $[M+2H-154]^{2+}$ as well

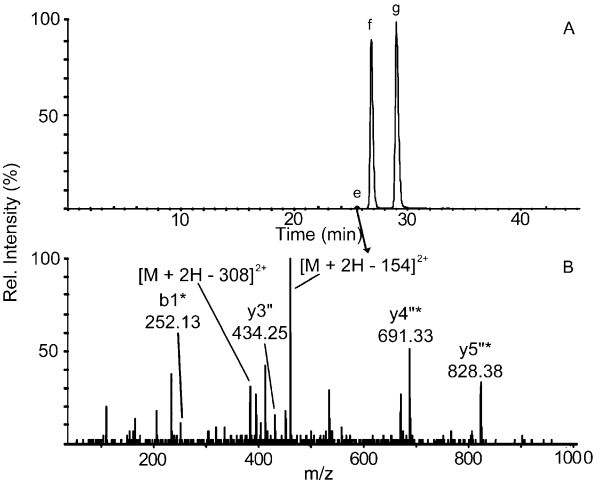

Figure 6. (A) Total-ion-current chromatogram obtained from product ion analysis of the doubly charged ion at m/z 540.27 of the hexapeptide H-Pro-His-Cys-Lys-Arg-Met-OH modified with two HHPA molecules in a phosphate buffer (pH 7.4), 21 h after synthesis. Analysis was performed with LC-QqTOFMS. (B) MS/MS mass spectrum of peak e contained data that localized the HHPA addition to the N-terminal proline and the cysteine residue **8** Pro*-His-Cys*-Lys-Arg-Met.

as the $[M+2H-308]^{2+}$ were present in the spectrum, indicating loss of one and two HHPA molecules from the peptide. The intensity of the peak at m/z 617.30, corresponding to the doubly charged hexapeptide with three HHPA adducts, was not high enough to allow sequencing.

The isolated adducts **5** were stable for several hours in solution at pH 7.4. However, some of the initially formed adducts, were slowly hydrolysed to the peptide and free HHP acid.

Transfer of HHPA-adduct from NAcCys to NAcLys

The reactions between NAcCys-HHPA **2** and NAcLys in a phosphate buffer (pH 7.4) were monitored with LC/MS/MS. The NAcCys-HHPA adducts were shown to react further in the presence of NAcLys and the stable NAcLys-HHPA amide adducts **3** were formed (m/z 343.2). Several experiments showed that the reaction was fast and the formation of NAcLys-HHPA levelled out within 12 min (Figures 7 and 8).

Discussion

The main finding was that HHPA bound to *N*-acetyl-L-cysteine could be transferred to the ε-amino group of N^{α}-acetyl-L-lysine at pH 7.4. We also identified stable HHPA adducts of lysine and the α-amino group of proline and semistable adducts with cysteine, histidine, tryptophan and tyrosine.

Allergic reactions of type IV, and some of type I, are thought to be mediated by hapten-modified proteins. In type I allergy, specific IgE antibodies are formed against these modified proteins, but in type I as well as the type IV allergy, the modified proteins are digested to peptides by antigen-presenting cells, which in turn present these peptides to T-cells. The binding sites of the haptens in these peptides have not been determined and it is unknown whether types I and IV allergy can be induced by identical epitopes. Consequently, in order to determine possible binding sites for the hapten during its diffusion through the skin, we investigated the capability of HHPA to form adducts with all nucleophilic amino acids at pH 5.5, representing the pH of the surface of the skin, and at pH 7.4, representing physiological pH.

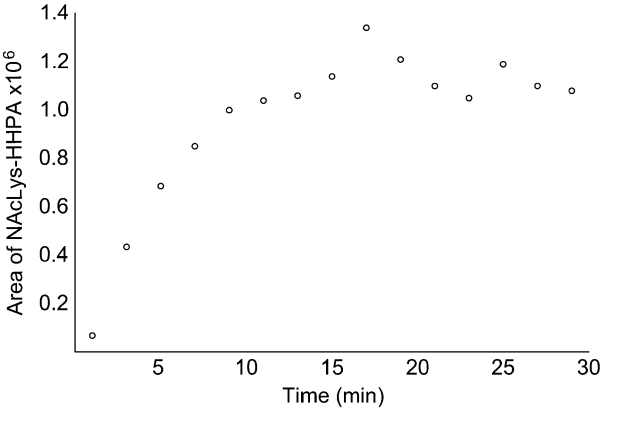

Figure 7. LC/MS/MS monitoring of the formation of N^{α}-acetyl-N^{ε}-hexahydrophthaloyl-L-lysine **3** in a 0.1 M phosphate buffer solution with *N*-acetyl-*S*-hexahydrophthaloyl-L-cysteine **2** and N^{α}-acetyl-L-lysine.

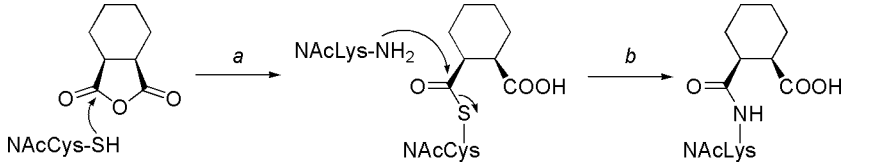

Figure 8. Reaction mechanism of the nucleophilic addition (a) of the thiol group of cysteine with HHPA (this mechanism also applies to the amino groups of proline and lysine), resulting in the reactive thio adduct **2**. This adduct then reacts further (b) with the ε-amino group of lysine forming the stable amide adduct **3**.

We found adduct formation between HHPA and the *N*-acylated amino acids cysteine histidine, lysine, tryptophan and tyrosine at pH 7.4. We also found addition of HHPA to the α-amino group of proline. The formation of anhydride adducts to cysteine, histidine, lysine and tyrosine is in accordance with the results of Palacian et al. (1990), whose considered the modifications generated by other dicarboxylic acid anhydrides on nucleophilic amino acids. However, Palacian et al. also reported stable anhydride adducts to serine. In the present study, no addition of HHPA to serine was detected. At pH 5.5, only cysteine and proline were capable of adduct formation with HHPA. It has been shown by Alvarez-Sánchez et al. (2004a) that sultones (type IV haptens) can add to histidine. Our results are in accordance with these findings, although the stability of the histidine–HHPA adduct was low.

The formation of the cysteine adducts is expected since this amino acid is known to be a strong nucleophile. For the formation of amides, the reactivity depends on the state of the nucleophilic amino group, since protonation should abolish these reactions. It is therefore somewhat surprising to find proline adducts formed to the extent shown in our experiments at pH 5.5. The pK_a for the secondary amine of proline is 10.6, but the reactivity of secondary amines can be higher than for primary amines. Thus, despite the low amount of proline available for adduct formation, a higher reactivity might compensate for this fact.

The stabilities were much higher for the amide adducts than for the adducts with ester bonds. Thus, the amide adducts were not hydrolysed like the ester or thio ester adducts of tyrosine and cysteine at pHs 5.5 and 7.4. It has previously been described that the HHPA–Hb adducts *in vivo* are stable during the lifetime of the red blood cells (Lindh & Jönsson 1998). *In vitro* studies have shown the major binding sites of HHPA to Hb to be lysine and the N-terminal valine (Lindh & Jönsson 1998, Kristiansson et al. 2002). These results are in agreement with those of the present study.

The model peptide used in this study contained several nucleophilic residues, and HHPA was found primarily to form stable adducts with the α-amino group of proline, even at pH 5.5. This is somewhat surprising, but the observation is in agreement with the result for the formation of proline adducts as described above. The detailed reaction mechanism of this formation may be proceeded by an initially formed thio adduct that reacts in the same way as the reaction of **2** with NAcLys (Figure 8). It was not possible to isolate HHPA bound to the cysteine residue in the peptide. However, such an adduct was detected by LC QqTOFMS. Furthermore, up to three HHPA bound to the peptide were detected, although the MS spectral data were not sufficient to determine the structure of the peptide with three HHPA bonded. However, the fact that three residues bound HHPA supports the identification of a cysteine as well as a lysine adduct of HHPA.

It is an important observation that the NAcCys–HHPA adduct formed initially could participate in a further reaction with NAcLys. Despite the high reactivity of HHPA, adducts to haemoglobin have been detected in exposed workers (Jönsson et al. 1997, Lindh & Jönsson 1998) as well as HHPA bound to tissue in the kidneys of an HHPA-exposed rat (Lindh et al. 1999). Since HHPA is quickly hydrolysed to HHP acid under physiological conditions, it has been suggested that the mechanism of transport to these distant sites is mediated by a reactive intermediate. *In vitro* studies of toluene di-isocyanate glutathione adducts and a peptide containing nucleophilic sites have shown that the isocyanate moiety could be transferred to the peptide by a glutathion adduct (Day et al. 1997). In a recent study (Alvarez-Sánchez et al. 2004b), a sultone was shown to give increased covalent binding to lysine residues in human serum albumin if glutathione was present. Our study suggests that formation of reactive thio adducts could be a general mechanism for the transport of some thiol reactive haptens to other sites in the living organism.

Hexahydrophthalic anhydrides are interesting compounds to study due to the dual types of allergies to which they give rise. The route of uptake of the anhydride may be essential for determining the type of allergy that will be generated. Our study has focused on the reactivity of HHPA and the HHPA adducts formed with different amino acids in proteins. However, the degradation of hapten-modified proteins in the skin, resulting in T-cell-specific immune response, can be different from the antigen formed in the airway mucosa. Synthetic complete antigens will be valuable tools in further studies to determine whether the antigenic epitopes formed in the skin and the airway are identical. The reaction mechanisms presented in our study give a good chemical base for further analytical, synthetic and biological investigation.

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