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# Influenza virus H5N1 hemagglutinin (HA) T-cell epitope conjugates: design, synthesis and immunogenicity

**Theodore Skarlas,[a‡] Stella Zevgiti,[a‡] Karoline Droebner,[b] Eugenia Panou-Pomonis,[a*] Oliver Planz[b] and Maria Sakarellos-Daitsiotis[a]**

**The influenza virus, major surface glycoprotein hemagglutinin (HA) is one of the principal targets for the development of protective immunity. Aiming at contributing to the development of a vaccine that remains the first choice for prophylactic intervention, a reconstituted model of HA, mimicking its antigenic properties was designed, synthesized and tested in mice for the induction of protective immunity. Four helper T lymphocyte [HTL ($T_1$, $T_3$, $T_7$ and $T_8$)] and four cytotoxic lymphocyte [CTL ($T_2$, $T_4$, $T_5$ and $T_6$)] epitopes were coupled in two copies each to an artificial carrier, $SOC_4$, which was formed by the repeating tripeptide Lys-Aib-Gly. The helical conformation of the $SOC_4$-conjugates preserves the initial topology of the attached epitopes, which is critical for their immunogenic properties. Survival of immunized animals, ranged from 30 to 50%, points out the induction of protective immunity by using the $SOC_4$-conjugates. Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.**

**Keywords:** H5N1; hemagglutinin (HA); T-cell epitopes of HA; immunogenic $SOC_4$-conjugates; protective immunity

## Introduction

Among emerging and re-emerging infectious diseases, influenza constitutes one of the major threats to mankind. Therefore, development of an H5N1 vaccine is recognized as the primary strategy to protect humans against a possible H5N1 pandemic. The influenza virus major surface glycoprotein hemagglutinin (HA) is one of the principal targets for the development of protective immunity. Peptides generated from influenza internal antigens, synthesized within the cells, are targets for cytotoxic lymphocytes (CTLs), which destroy infected cells presenting the peptide-major histocompatibility (MHC) class I complexes. Recent studies demonstrated that HA-specific antibodies are primarily responsible for preventing infection, while CTLs are responsible for reducing viral spread and thereby for accelerating the recovery from influenza [1–4].

Formulation of an effective immunogen and consequently a vaccine that could elicit a potent immune response should incorporate B-cell epitopes that mimic the three-dimensional conformation of the antigen and T-cell epitopes resulting from the processed protein, both on the same artificial carrier that links the MHC complex with the T-cell receptor. In fact, identification of CD8+ and CD4+ epitopes remains a challenge because of the MHC polymorphism and the antigenic variation in influenza viruses [5–8].

A great variety of artificial carriers encompassing epitopes with multiple functionalities appeared in the literature during the past decades for the construction of antigenic and immunogenic conjugates as diagnostics and vaccine candidates.

Sequential oligopeptide carrier ($SOC_n$, $n = 2–7$), a novel foldamer class of artificial carriers formed by the repeating tripeptide unit Lys-Aib-Gly, was introduced by our laboratory with the aim of optimizing the presentation of the anchored epitopes, to the Lys-$N^{\varepsilon}H_2$ groups, and help in the reconstitution of immunogenic/antigenic protein mimics. $SOC_n$ is a sterically constrained scaffold, characterized by a few conformations in solution, comprising $\alpha$-aminoisobutyric acid (Aib), a strongly helicogenic $C^{\alpha}$-tetrasubstituted amino acid. It was found that the helical conformation of the tetrameric $SOC_4$ attributed mainly to the inclusion of Aib, induces a favorable arrangement of the conjugated epitopes, which retain their initial 'active' conformation and their potential for generating site-specific immune responses [9–12].

Predictions of MHC class I binding peptides (CTL epitopes) and class II binding peptides [helper T lymphocytes (HTL) epitopes] allowed the selection of top scoring peptide epitopes, which were used for the synthesis of $SOC_4$-conjugates and their study as immunogens [7,13]. T-cell epitopes from HA of H5 VLMENERTL($T_1$), VWTYNAELL($T_2$), SFFRNVVWL($T_3$), YNNTNQEDL($T_4$), FHDSNVKNL($T_5$), QLRDNAKEL($T_6$), NFESNGNFI($T_7$) and MPFHNIHPL($T_8$) were coupled in two copies each to the first and third Lys-$N^{\varepsilon}H_2$ group of the carrier $SOC_4$. The couplings were performed according to the chemoselective ligation approach, which generates an oxime bond between the $H_2N–O–$groups of the modified lysine residues and the aldehyde groups of the modified T-cell epitopes (Figure 1).

* *Correspondence to: Eugenia Panou-Pomonis, Department of Chemistry, University of Ioannina, 45110 Ioannina, Greece. E-mail: epanou@cc.uoi.gr*

a *Department of Chemistry, University of Ioannina, 45110 Ioannina, Greece*

b *Institute of Immunology, Fridrich-Löffler Institut, Tübingen, Germany*

‡ *Theodore Skarlas and Stella Zevgiti contributed equally to this work.*

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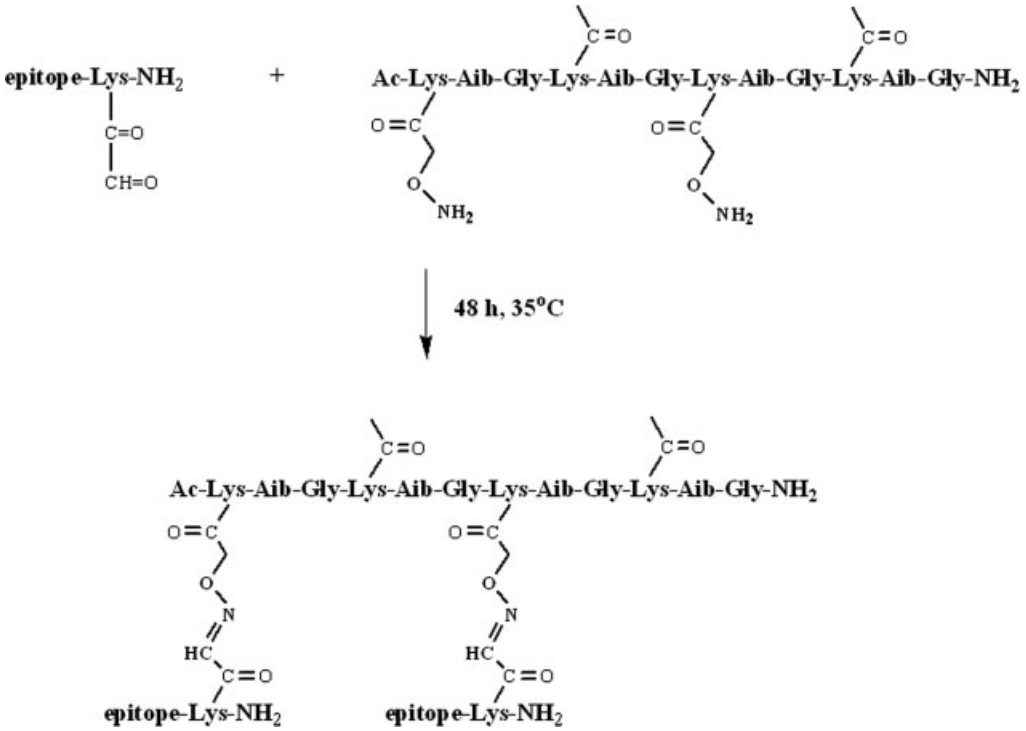


**Figure 1.** Chemoselective oxime ligation of the aldehyde T-cell epitope to the Ac–$SOC_4[(Ac)_2,(Aoa)_2]$–$NH_2$ (**9**).

Immunization experiments, using the precedent $SOC_4$-conjugates, induced protective immunity in mice. It is concluded that the synthesized conjugates are successful reconstituted mimics of the HA antigen and that the beneficial arrangement of the coupled T-cell epitopes to the carrier preserves their potential for the induction of specific immunity.

## Materials and Methods

### Peptide Synthesis

SPPS was carried out manually following the Fmoc/tBu methodology on a Rink Amide AM resin (0.67 mmol/g). Fmoc groups were removed using 20% piperidine/DMF. Coupling of each Fmoc-amino acid (3 mol equiv.) was performed in the presence of O-Benzotriazole-*N*, *N*, *N*′, *N*′-tetramethyl-uronium-hexafluoro-phosphate (HBTU)/HOBt/DIEA (2.9/3/6 molar ratio) in a DMF/DCM mixture. Completion of couplings and deprotection reactions were monitored using the Kaiser ninhydrin test. The synthesized compounds are shown in Tables 1 and 2.

### Synthesis of the T-Cell Epitopes and Formation of the Aldehyde Groups

The synthesis of the T-cell epitopes was carried out using the SPPS methodology on a Rink Amide AM resin [14,15]. The amino acids were added as Fmoc-protected derivatives with suitable side chain protections when necessary. The *C*-terminus of each T-cell epitope was elongated by the -Lys(Ser) motive to create an aldehyde group. The Lys and Ser residues were introduced as Fmoc-Lys(Mtt)-OH and Fmoc-Ser(tBu)-OH, respectively. After completion of the synthesis the Mtt group was removed by 1.8% TFA/DCM and Fmoc-Ser(tBu)-OH was coupled to the Lys-$N^{\varepsilon}H_2$ group using DIC and HOBt (3 molar excess). Fmoc groups of the *N*-terminus and the seryl amino group were removed followed by cleavage of the epitope from the resin and side chain deprotection after treatment with TFA/triisopropylsilane (TIS)/$H_2O$ (95/2.5/2.5, v/v/v, 4h). Resin was removed by filtration, the filtrate was evaporated under reduced pressure and the product was precipitated with cold diethyl ether. The precipitate was filtrated, dissolved in 2N aqueous acetic acid

**Table 1.** Synthesized aldehyde T-cell epitopes **1**–**8**

| Number | Amino acid sequences |
|---|---|
| **1** | VLMENERTLK(CHOCO)A–$NH_2$ ($T_1$) |
| **2** | VWTYNAELLK(CHOCO)–$NH_2$ ($T_2$) |
| **3** | ASFFRNVVWLK(CHOCO)–$NH_2$ ($T_3$) |
| **4** | YNNTNQEDLK(CHOCO)–$NH_2$ ($T_4$) |
| **5** | FHDSNVKNLK(CHOCO)–$NH_2$ ($T_5$) |
| **6** | QLRDNAKELK(CHOCO)–$NH_2$ ($T_6$) |
| **7** | NFESNGNFIK(CHOCO)–$NH_2$ ($T_7$) |
| **8** | MPFHNIHPLK(CHOCO)–$NH_2$ ($T_8$) |

**Table 2.** Synthesized carrier and $SOC_4$-conjugates **9**–**17**

| Number | Carrier and $SOC_4$-conjugates |
|---|---|
| **9** | Ac–$SOC_4(Ac_2, Aoa_2)$–$NH_2$ |
| **10** | Ac–$SOC_4\{Ac_2, [T_1(CH{=}N{-}O)]_2\}$–$NH_2$ |
| **11** | Ac–$SOC_4\{Ac_2, [T_2(CH{=}N{-}O)]_2\}$–$NH_2$ |
| **12** | Ac–$SOC_4\{Ac_2, [T_3(CH{=}N{-}O)]_2\}$–$NH_2$ |
| **13** | Ac–$SOC_4\{Ac_2, [T_4(CH{=}N{-}O)]_2\}$–$NH_2$ |
| **14** | Ac–$SOC_4\{Ac_2, [T_5(CH{=}N{-}O)]_2\}$–$NH_2$ |
| **15** | Ac–$SOC_4\{Ac_2, [T_6(CH{=}N{-}O)]_2\}$–$NH_2$ |
| **16** | Ac–$SOC_4\{Ac_2, [T_7(CH{=}N{-}O)]_2\}$–$NH_2$ |
| **17** | Ac–$SOC_4\{Ac_2, [T_8(CH{=}N{-}O)]_2\}$–$NH_2$ |



  

and after lyophilization the obtained crude epitope was purified by semipreparative RP-HPLC. Oxidation of the 2-amino-alcohol group of serine (coupled to the *C*-terminus Lys-N$^{\varepsilon}$H$_2$ group) by $NaIO_4$ resulted in the formation of an aldehyde group: Aqueous solution of the epitope (10 mM) was diluted with imidazole buffer (50 mM, pH 7 and chloride counter-ion) and $NaIO_4$ (12 mM) was added in the mixture. After 4 min, the oxidation was quenched by adding ethyleneglycol solution (100 mM) [16–18]. The epitopes were isolated by semipreparative RP-HPLC and characterized by analytical RP-HPLC and ESI-MS (Tables 1 and 3).

### Synthesis of the Carrier Ac–SOC$_4$[(Ac)$_2$,(Aoa)$_2$]–NH$_2$(9)

The synthesis of $SOC_4$ was carried out on a Rink Amide AM resin by the SPPS procedure [14,15]. Lysines at the 4th and 10th positions were introduced as Fmoc-Lys(Ac)-OH, whereas lysines at the 1st and 7th positions were introduced as Fmoc-Lys(Mtt)-OH. The *N*-terminal Fmoc group of the carrier was removed and the free $\alpha$-amino group was acetylated using acetic anhydride in pyridine. Subsequently, removal of the Mtt group from the two Lys-N$^{\varepsilon}$H$_2$ groups was followed by coupling to Boc-aminooxy-acetic acid using DIC and HOBt (3 molar excess). Removal of Boc protective group and removal from the resin were carried out using TFA/TIS/$H_2O$ (95/2.5/2.5, v/v/v, 4h). Resin was removed by filtration, the filtrate was evaporated under reduced pressure and the product was precipitated with cold diethyl ether. The precipitate was filtrated, dissolved in 2N aqueous acetic acid and after lyophilization the obtained crude peptide was purified by semipreparative RP-HPLC and characterized by analytical RP-HPLC and ESI-MS (Tables 2 and 4), (Figure 2).

### Chemoselective Ligation of the Modified T-Cell Epitopes (1–8) with the Ac–SOC$_4$[(Ac)$_2$,(Aoa)$_2$]–NH$_2$(9)

$SOC_4$-conjugates **10**–**17** (Table 2) were synthesized in the liquid phase using the chemoselective ligation approach which leads to the formation of an oxime bond between each aminooxy group of the $SOC_4$ carrier (**9**) and the corresponding aldehyde-epitope. Oxime reaction was initiated by mixing 250 µl of the carrier (10 mM in water) with 5.25 ml of each aldehyde-epitope (1.43 mM in water: 0.1 M acetate buffer, pH 4.6, 1 : 6 v/v) at 35 °C (Figure 1). The aldehyde-epitope was present in a 1.5-fold molar excess over each reactive group on the carrier. Reaction progress was followed by ESI-MS and the product was purified by semipreparative HPLC and characterized by analytical HPLC and ESI-MS [16–18] (Tables 2 and 4), (Figure 3).

**Table 3.** Parameters of the synthesis, purification and characterization of the compounds **1**–**8**

| Peptide | Yield (%)[a] | RP-HPLC gradient elution[b] | $t_R$ (min) | ESI-MS |
|---|---|---|---|---|
| **1** | 68 | A/B: 90 : 10 | 12 | Calculated $[M+H]^+$: 1359.6 |
| | | A/B: 30 : 70 | | Found $[M+H]^+$: 1359.8 |
| **2** | 71 | A/B: 90 : 10 | 19.5 | Calculated $[M+H]^+$: 1292.5 |
| | | A/B: 50 : 50 | | Found $[M+H]^+$: 1292.8 |
| **3** | 66 | A/B: 90 : 10 | 20.5 | Calculated $[M+H]^+$: 1422.7 |
| | | A/B: 30 : 70 | | Found $[M+H]^+$: 1423.3 |
| **4** | 65 | A/B: 90 : 10 | 6.5 | Calculated $[M+H]^+$: 1294.3 |
| | | A/B: 50 : 50 | | Found $[M+H]^+$: 1294.5 |
| **5** | 70 | A/B: 90 : 10 | 12.5 | Calculated $[M+H]^+$: 1257.4 |
| | | A/B: 50 : 50 | | Found $[M+H]^+$: 1257.2 |
| **6** | 67 | A/B: 90 : 10 | 12 | Calculated $[M+H]^+$: 1270.4 |
| | | A/B: 50 : 50 | | Found $[M+H]^+$: 1270.9 |
| **7** | 70 | A/B: 90 : 10 | 13.5 | Calculated $[M+H]^+$: 1225.3 |
| | | A/B: 40 : 60 | | Found $[M+H]^+$: 1225.7 |
| **8** | 63 | A/B: 90 : 10 | 17 | Calculated $[M-H]^-$: 1287.5 |
| | | A/B: 50 : 50 | | Found $[M-H]^-$: 1287.5 |

[a] The purity of the final products was analyzed according to HPLC peak integrals at 214 nm on analytical HPLC and was estimated >95%.
[b] A: $H_2O$/0.1% TFA, B: $CH_3CN$/0.1% TFA.

### Analytical RP-HPLC

Analytical HPLC of the synthesized compounds (**1**–**17**) was performed using a Supelco Discovery (Sigma Aldrich, USA) C18 (25 cm × 4.6 mm, 5 µM) RP column with a Waters instrument equipped with a Waters 616 pump and a Waters 2487 dual λ absorbance detector. Eluent A was 0.1% TFA in water and eluent B was 0.1% TFA in acetonitrile. A linear gradient was used from 10 to 70% acetonitrile in 0.1% TFA at a flow rate of 1 ml/min.

### Semipreparative RP-HPLC

RP-HPLC purification was carried out on a SHIMADZU (Shimadzu Corporation, Japan) semipreparative instrument equipped with a SHIMADZU LC-10AD VP pump and a SHIMADZU SPD-10A VP UV-VIS detector, using a Supelco Discovery C18 (25 cm × 10 mm, 5 µM) column, with the same linear gradient as for analytical RP-HPLC at a flow rate of 4.7 ml/min. Detection was carried out at λ = 214 nm. Acetonitrile was evaporated and the fractions were lyophilized to obtain the pure compounds.

**Table 4.** Parameters of the synthesis, purification and characterization of the carrier $SOC_4$ and the $SOC_4$-conjugates **9**–**17**

| Number | Yield (%)[a] | RP-HPLC gradient elution[b] | $t_R$ (min) | ESI-MS |
|---|---|---|---|---|
| **9** | 38 | A/B: 90 : 10 | 13 | Calculated $[M+H]^+$: 1371.6 |
| | | A/B: 50 : 50 | | Found $[M+H]^+$: 1372.4 |
| **10** | 48 | A/B: 90 : 10 | 17 | Calculated $[M+3H]^{+3}$: 1351.6 |
| | | A/B: 30 : 70 | | Found $[M+3H]^{+3}$: 1352.0 |
| **11** | 52 | A/B: 90 : 10 | 25 | Calculated $[M+4H]^{+4}$: 980.4 |
| | | A/B: 50 : 50 | | Found $[M+4H]^{+4}$: 980.8 |
| **12** | | Dialysis | – | Calculated $[M+4H]^{+4}$: 1045.5 |
| | | | | Found $[M+4H]^{+4}$: 1046.0 |
| **13** | 67 | A/B: 90 : 10 | 15.5 | Calculated $[M+4H]^{+4}$: 981.3 |
| | | A/B: 50 : 50 | | Found $[M+4H]^{+4}$: 981.8 |
| **14** | 66 | A/B: 90 : 10 | 18 | Calculated $[M+4H]^{+4}$: 962.8 |
| | | A/B: 40 : 60 | | Found $[M+4H]^{+4}$: 963.3 |
| **15** | 33 | A/B: 90 : 10 | 20.5 | Calculated $[M+4H]^{+4}$: 969.4 |
| | | A/B: 50 : 50 | | Found $[M+4H]^{+4}$: 969.8 |
| **16** | 37 | A/B: 90 : 10 | 17.8 | Calculated $[M+3H]^{+3}$: 1262.1 |
| | | A/B: 40 : 60 | | Found $[M+3H]^{3+}$: 1262.6 |
| **17** | 60 | A/B: 90 : 10 | 18.5 | Calculated $[M+3H]^{+3}$: 1304.9 |
| | | A/B: 50 : 50 | | Found $[M+3H]^{+3}$: 1305.1 |

[a] The purity of the final products was analyzed according to HPLC peak integrals at 214 nm on analytical HPLC and was estimated >95%.
[b] A: $H_2O$/0.1% TFA, B: $CH_3CN$/0.1% TFA.



  

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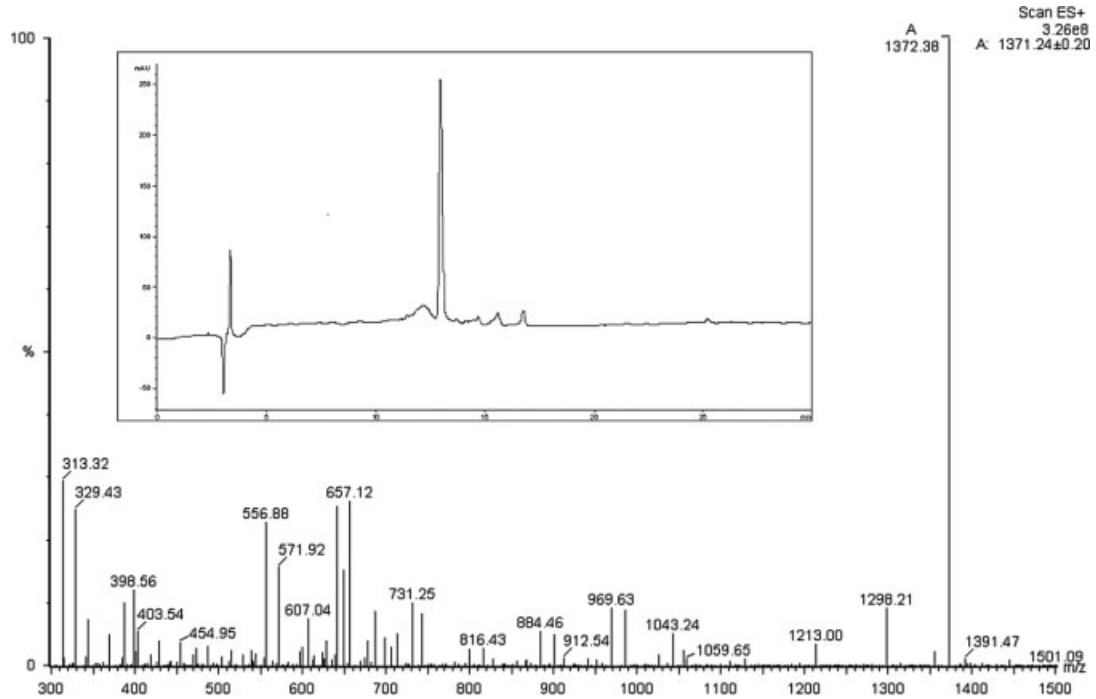


**Figure 2.** ESI-MS and analytical RP-HPLC of the purified carrier Ac–$SOC_4[(Ac)_2,(Aoa)_2]$–$NH_2$ (**9**).

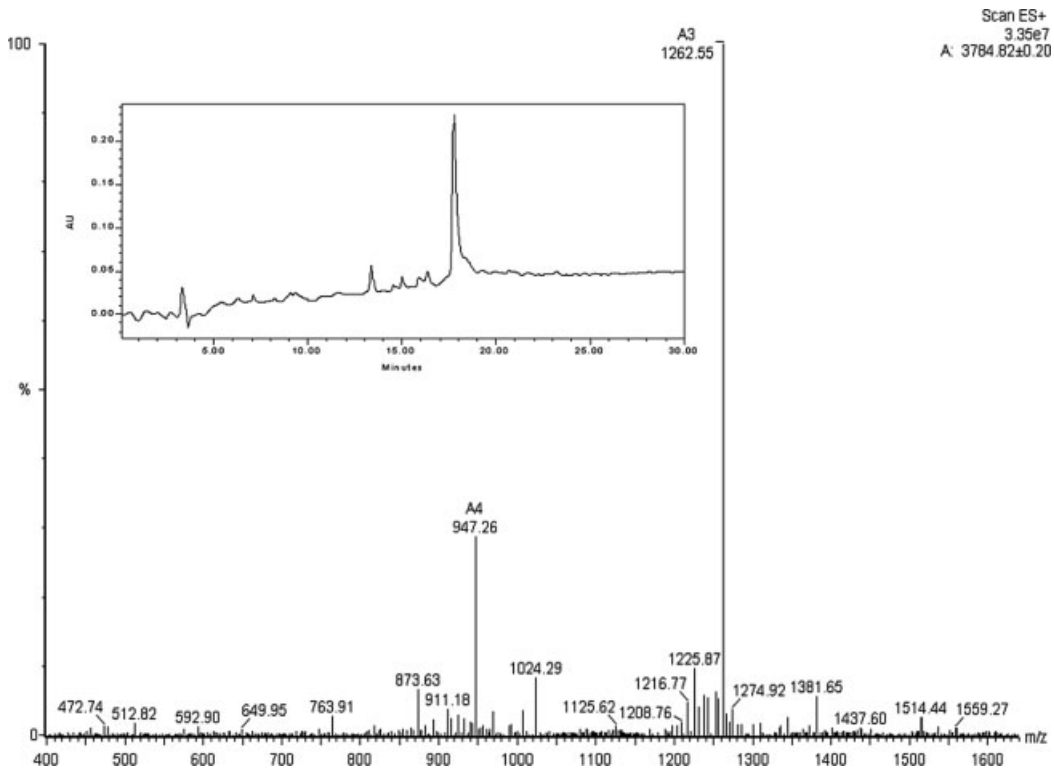


**Figure 3.** ESI-MS and analytical RP-HPLC of the purified Ac–$SOC_4\{Ac_2, [T_7(CH{=}N{-}O)]_2\}$–$NH_2$ conjugate (**16**).

### Mass Spectrometry

Positive or negative ion ESI-MS analyses of synthesized compounds **1**–**17** were performed on a micromass platform LC–MS. Capillary and cone voltages were set to 3 kV and 35–75 V, respectively. Samples were dissolved in water/acetonitrile/trifluoroacetic acid (1/1/0.05 v/v/v). Molecular ions calculated and found are given in Tables 3 and 4.

### Circular Dichroism

CD spectra were recorded at 25 °C on a Jasco J-815 (Jasco Corporation, Japan) spectropolarimeter equipped with a thermoelectric



  

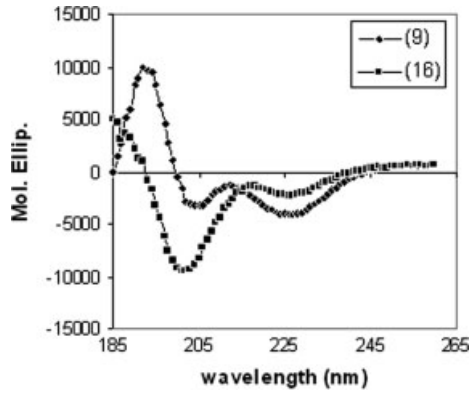


**Figure 4.** CD spectra of the carrier Ac–$SOC_4$[$(Ac)_2$,$(Aoa)_2$]–$NH_2$ (**9**) and the Ac–$SOC_4${$Ac_2$, [$T_7$(CH═N–O)]$_2$}–$NH_2$ conjugate (**16**) in 50% TFE/$H_2O$.

**Table 5.** Animal immunizations with $SOC_4$-conjugates

| Immunized mice | Died | Survived |
|---|---|---|
| Ac–$SOC_4${$Ac_2$, $Aoa_2$}–$NH_2$ (**9**) | 6 | 0 |
| Ac–$SOC_4${$Ac_2$, [$T_1$(CH═N–O)]$_2$}–$NH_2$ (**10**) | 5 | 1 |
| Ac–$SOC_4${$Ac_2$, [$T_2$(CH═N–O)]$_2$}–$NH_2$ (**11**) | 5 | 1 |
| Ac–$SOC_4${$Ac_2$, [$T_3$(CH═N–O)]$_2$}–$NH_2$ (**12**) | 6 | 0 |
| Ac–$SOC_4${$Ac_2$, [$T_4$(CH═N–O)]$_2$}–$NH_2$ (**13**) | 4 | 2 |
| Ac–$SOC_4${$Ac_2$, [$T_5$(CH═N–O)]$_2$}–$NH_2$ (**14**) | 4 | 2 |
| Ac–$SOC_4${$Ac_2$, [$T_6$(CH═N–O)]$_2$}–$NH_2$ (**15**) | 6 | 0 |
| Ac–$SOC_4${$Ac_2$, [$T_7$(CH═N–O)]$_2$}–$NH_2$ (**16**) | 3 | 3 |
| Ac–$SOC_4${$Ac_2$, [$T_8$(CH═N–O)]$_2$}–$NH_2$ (**17**) | 6 | 0 |
| Adjuvant | 6 | 0 |
| Control | 6 | 0 |

temperature controller. Spectra were obtained using a quartz cell of 1 mm path length and the concentration of the tested compounds was 100 μM or 50 μM. Experiments were performed in TFE/$H_2O$ mixtures (0, 50 and 100%). Spectra were obtained with a 1 nm bandwidth, a scan speed of 50 nm/min and a response of 1 s. The signal-to-noise ratio was improved by accumulating three scans. All CD spectra are reported in terms of ellipticity units per mole ([Θ] in deg $cm^2$ $dmol^{-1}$). The percentage helical content was estimated on the basis of the $[\Theta]_{222}$ nm values, in different environments, as described by Chen *et al*. [19] (Figure 4).

### Biological Assays

The animal experiments were performed in the laboratories of the co-authors Droebner and Planz at the Friedrich-Loeffler-Institut (FLI), Tuebingen, Germany. The FLI is an unique 'high-safety' BSL3 building where experiments with wild-type H5N1 influenza virus are allowed to be performed. The experiments were performed with an original field isolate from a mallard that is highly pathogenic to mice without adaptation to the mammalian host. The carrier (**9**) and the $SOC_4$-conjugates (**10**–**17**) were dissolved in $ddH_2O$. Immunizations were performed according to Papamattheou *et al*. [20]. Groups of six female bagg albino (BALB)/c mice (at an age of 6–8 weeks) were immunized subcutaneously (s.c.) with 50 μg of each $SOC_4$-conjugate dissolved in $ddH_2O$ and emulsified in complete Freund's adjuvant (CFA) on day 0. Two boosters of half-doses of each $SOC_4$-conjugate in incomplete Freund's adjuvant (IFA) followed at days 25 and 50. Twenty-five days past the third boost, the animals were infected intranasally with $2 \times 10^5$ pfu (100× $MLD_{50}$) H5N1 (A/mallard/Bavaria/1/2006, MB1) influenza virus. The survival rates were determined after a 14-day period [21]. The experiments were performed twice.

## Results and Discussion

Although avian influenza has been extensively studied in the past 30 years, our knowledge of the immune response to this pathogen remains rather limited. Vaccination is the most efficient method for preventing influenza and its severe complications. However, antigenic drift has a major impact on the vaccine effectiveness [22] and the development of a universal influenza vaccine is still at a preclinical or clinical phase. Current avian influenza vaccines are divided into whole viral vaccines and subunit vaccines. The whole virus vaccines primarily include only the killed vaccines that are inactivated. Subunit vaccines can be used in a variety of formats, but typically they all are targeted to the HA gene [23].

Both antibodies and activated T lymphocytes were produced in response to viral infection. There are reports indicating that influenza virus-specific IgG response is CD4+ T-cell dependent and others describing CD4+ T-cell independent antibody responses. CTL response is considered to be directed against the M and NP proteins, while other investigations pointed out that CTL response in addition to strong antibody formation is more effective in preventing the disease [21,24–26].

Aiming at contributing to the development of a vaccine that remains the first choice for prophylactic intervention, a reconstituted model of HA, mimicking its antigenic properties was designed, synthesized and tested in mice for the induction of protective immunity. Four HTL ($T_1$, $T_3$, $T_7$ and $T_8$) and four CTL ($T_2$, $T_4$, $T_5$ and $T_6$) epitopes were coupled in two copies each to the tetrameric $SOC_4$, (Lys-Aib-Gly)$_4$, carrier. The $SOC_4$-conjugates were synthesized in three steps: (i) solid phase synthesis of the $SOC_4$ carrier bearing two amino-oxy-acetyl ($NH_2$–$OCH_2CO$) groups on the first and third Lys-$N^{\varepsilon}H_2$ residues, (ii) solid phase synthesis of each epitope and creation of an aldehyde group and (iii) chemoselective ligation of the aldehyde-epitopes to the carrier through the formation of an oxime bond. All final products were obtained in sufficient yields and high purity as confirmed by HPLC and ESI-MS (Tables 3 and 4).

The CD spectra of the amino-oxy-acetyl $SOC_4$ carrier, Ac–$SOC_4$[$(Ac)_2$,$(Aoa)_2$]–$NH_2$ (**9**), in TFE/$H_2O$ (50/50 v/v) and 100% TFE exhibited a positive band at 192 nm and two negative bands at 205 and 222 nm typical of helical structure. The helical content of the carrier, estimated on the basis of the $[\Theta]_{222}$ value, was found to be 15% at 50% TFE/$H_2O$ [19]. These helical features, in agreement with our previous studies, were conserved even after the attachment of the T-cell epitopes to the carrier, as for example in the case of Ac–$SOC_4${$Ac_2$, [$T_7$(CH═N–O)]$_2$}–$NH_2$ (**16**) confirming the persistence of the carrier-helical conformation in its conjugated forms (Figure 4). One may assume that the coupled T-cell epitopes of the HA protein will conserve their initial topology, which is critical for expressing their immunogenic properties.

Immunization experiments (Table 5) indicated 50% survival of mice when immunized with the $SOC_4$-conjugate bearing two copies of $T_7$ (**16**) and 30% survival when immunized with conjugates bearing two copies of $T_4$ (**13**) and $T_5$ (**14**) (Figure 5). Previous biological studies [21], performed in the same laboratory under comparable conditions, showed 60% survival of mice. It is



  

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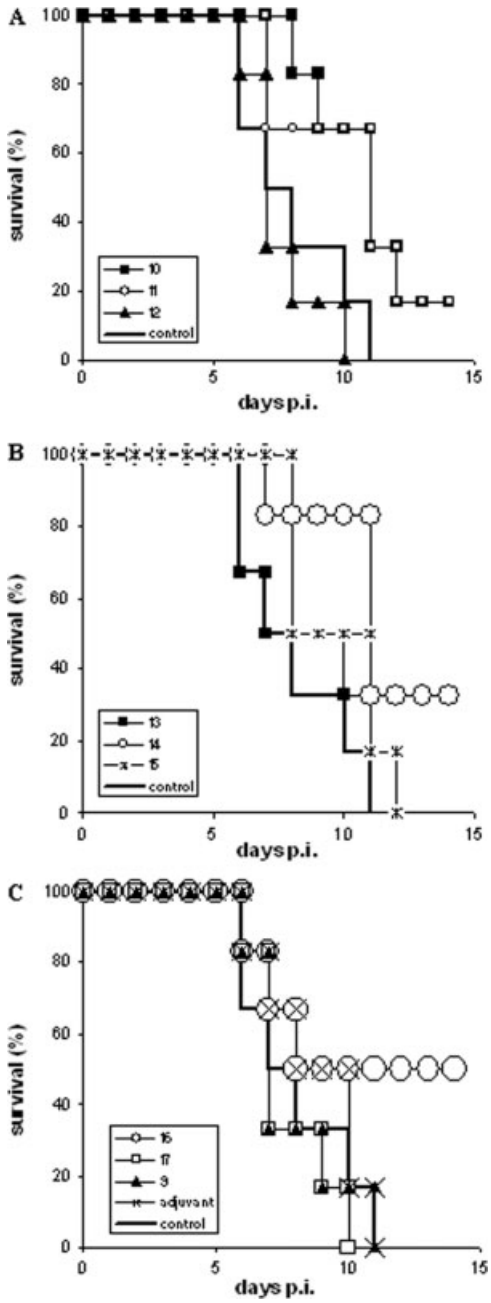


**Figure 5.** Survival curves indicating the days that the mortality occurred for each mice group immunized with the corresponding $SOC_4$-conjugate. (A) Immunizations with $SOC_4$-conjugates **10**–**12**. (B) Immunizations with $SOC_4$-conjugates **13**–**15**. (C) Immunizations with $SOC_4$-conjugates **16, 17**, with $SOC_4$-carrier **9** and with adjuvant only. Control curve represents a non-immunized mice group.

concluded that the precedent conjugates can be applied for the induction of protective immunity. In particular, $SOC_4$-conjugate bearing two copies of $T_7$ could be a potential vaccine candidate.

## Conclusions

T-cell epitopes of H5N1 HA were coupled to the Lys-$N^{\varepsilon}H_2$ groups of an artificial carrier $(Lys\text{-}Aib\text{-}Gly)_4$, $SOC_4$, to formulate reconstituted mimics of the HA antigen. Synthesis was carried out successfully by the combined application of SPPS and chemoselective ligation. The helical features of the carrier, confirmed by CD, were conserved even after the attachment of the T-cell epitopes to the carrier. It is likely that the helical conformation of the $SOC_4$-conjugates preserves the initial topology of the attached epitopes, which is critical for their immunogenic properties. Immunization experiments indicated that $SOC_4$-conjugates bearing two copies each of $T_7$, $T_4$ and $T_5$ epitopes generate specific immune responses for the induction of protective immunity and the development of vaccine candidates.

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