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Structure–immunogenicity relationship of kresoxim-methyl regioisomeric haptens

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Kresoxim-methyl was one of the two first strobilurins to be discovered, and nowadays it is widely used as antifungal agent in crop protection. Because of its low molecular weight and little structural complexity, generation of antibodies to kresoxim-methyl noticeably requires the preparation of functionalized haptens. In this study, the introduction of a hydrocarbon spacer arm at aromatic moieties of the target molecule was carried out by a convergent strategy based on the Sonogashira cross-coupling reaction, and functionalized linkers of the same length were also tethered to the aliphatic toxophore group by *O*-alkylation reaction. Evaluation of the immune response, in terms of antibody affinity, showed a differential behavior among five synthesized haptens whose sole dissimilarity was the derivatization site. The characteristic (methoxyimino)acetate moiety of strobilurins was revealed as the optimum linker position for high-affinity polyclonal and monoclonal antibody production. However, good monoclonal antibodies were isolated from mice immunized with a hapten carrying the linker at an opposite site, which otherwise generated a poor polyclonal response in rabbits. Site-heterology was confirmed as a feasible approach for the improvement of the apparent affinity, particularly with polyclonal antibodies. Several of the monoclonal antibodies generated in the context of this project could be proper binders for kresoxim-methyl immunosensing over different analytical platforms.

Introduction

Strobilurins are one of the most important families of modern crop protection compounds. Their invention was made possible after the discovery and chemical description, by Anke and coworkers in 1977,¹ of strobilurin A – a natural antibiotic derivative of β -methoxyacrylic acid that was isolated from *Strobilurus tenacellus* and *Oudemansiella mucida*. More recently, it was discovered that strobilurins are also produced by other genera, such as *Xerula*, *Hydropus*, *Mycena*, *Filoboletus*, *Crepidotus*, *Cyphellopsis*, and *Bolinea*.² However, the naturally secreted product is not stable to light and therefore not useful for pest control. All currently registered strobilurins – 19 so far – are synthetic nature-derived chemicals, which are comprised of a common aromatic bridge that provides photochemical stability to the molecule and holds both the toxophore moiety and a distinctive bulky substituent in *ortho* position.³ Kresoxim-methyl, which was released in the mid 1990's by BASF, belongs to the first-generation of strobilurins. Structurally, kresoxim-methyl is characterized by a methyl (*E*)-2-(methoxyimino)-2-phenylacetate group (a-ring), which holds the toxophore moiety responsible of its biocide activity and an *o*-tolyl group (b-ring) – both rings being joined together by means of a methylidenoxy group (Fig. 1). At 20 °C, kresoxim-methyl is a white solid with low solubility in water (2 mg L⁻¹) and low volatility (vapor pressure of 1.72×10^{-8} mm Hg), and it is highly photostable showing a half-life time of 30 days. The synthetic pathway to prepare this molecule has evolved from the first procedure described by Wenderoth et

al. in 1989,⁴ which implied several synthetic steps for the construction of the (methoxyimino)acetate moiety, to more recent strategies based on the Suzuki–Miyaura coupling reaction that allow the stereoselective construction of the (*E*)-2-(methoxyimino)-2-phenylacetate moiety with high yield in only one synthetic step.⁵

Antibody-based techniques constitute nowadays valuable alternative strategies for small organic chemical monitoring. In particular, antibodies recognizing modern and relevant pesticides are deemed valuable tools for the development of rapid analytical methods enabling a more efficient control of chemical residues in foodstuffs and the environment. Since low molecular weight compounds like kresoxim-methyl are not able to trigger an immune response by themselves (so-called haptens), immunochemical methods rely on the availability of appropriate bioconjugates. Prior to antibody production, a rational design of functionalized haptens for covalent coupling to carrier proteins has long been recognized to be a critical step for high-quality antibody generation and sensitive assay development.^{6–10} Hapten functionalization aims at synthesizing an analogue of the target compound which holds a spacer arm with a reactive or activatable chemical group at the free end of the linker. Thus, several criteria need to be considered before haptens are prepared, such as the functional moiety, the length and composition of the linker, and the derivatization site that will be most adequate for optimum immune response. For the functional group, carboxylates are mostly preferred because they can easily be protected and deprotected for chemical synthesis, and they can be readily activated for high-yield covalent coupling to the abundantly

available ϵ -amine groups of lysine residues in proteins. The importance of linker composition and length has been investigated by our group and others.¹¹⁻¹⁹ Generally, linear aliphatic hydrocarbon chains are preferred due to their low immunogenicity, and medium-size spacers (four to six-carbon long) are mostly recommended for the generation of high-affinity and selective antibodies,^{20, 21} although other linker lengths and

compositions have also been employed.²²⁻²⁶ Last but not least, the linker tethering site decides the ultimate orientation of the hapten, thus determining upon immunization the formation of the antibody binding site, and consequently influencing detectability and selectivity.²⁷⁻³⁴

Fig. 1. A) Global minimum energy conformation of kresoxim-methyl. Calculations were performed using Molecular Mechanics (MM3) as implemented in the CAChe program. A systematic conformational search was performed (all rotatable bonds were rotated by 15 degree steps) and the geometry of the generated conformers was refined by performing an optimized geometry calculation in MOPAC using PM3 parameters. Arrows denote the spacer-arm attachment site in each of the haptens. B) Structure of kresoxim-methyl (KM), the synthesized haptens, and the corresponding *N*-succinimidyl esters (activated haptens).

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Functionalized haptens for kresoxim-methyl were previously prepared by our group through derivatization at the carboxyl group of the (methoxyimino)acetate moiety, using as linkers dipeptides or linear aliphatic chains of different lengths.¹² In the present study, a thorough approach has been undertaken in order to investigate the effect of the derivatization site on the generation of high-quality antibodies for this chemical. Five regioisomeric haptens – carrying identical spacers – were prepared with alternative rationally-chosen linker tethering sites, thus covering a wide variety of spatial orientations of the target molecule. A comprehensive study was undertaken so as to clarify the influence of hapten orientation over the immune system response. Rabbit polyclonal antibodies (pAb) and mouse monoclonal antibodies (mAb) were generated in order to evaluate the antibody-eliciting capability of the different haptens. Antibody apparent affinity was first estimated using homologous enzyme-linked immunosorbent assay (ELISA), and finally the influence of tethering-site heterology was assessed for each antibody/hapten combination.

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Results and discussion

Hapten syntheses

The kresoxim-methyl framework exhibited significant conformational flexibility due to the large number of bonds displaying free rotation. Calculations showed that, like natural strobilurins and several of its synthetic analogues,³⁵ kresoxim-methyl does not adopt an extended geometry, so all minimum-energy conformations are characterized by the nearly orthogonal disposition of the two planar phenyl moieties (see Fig. 1A for the most stable conformation). Particularly relevant is the high degree of conformational flexibility of the (methoxyimino)acetate moiety, which can adopt several energetically equivalent orientations by rotation around the C–C bond that connects it to the aromatic backbone. On the basis of the above conformational analysis, five different positions of the kresoxim-methyl framework were selected for incorporation of the linker, trying to cover as many spatial orientations of the target molecule as possible (Fig. 1A and 1B). All of the designed hapten structures

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maintained the complete hydrocarbon skeleton and functional groups of the target analyte, incorporating the same aliphatic spacer arm at positions where minimum modifications of

electronic, steric, and conformational features of the molecule were expected.

Fig. 2. Synthetic sequence for the preparation of haptens KMa, KMb, and KMc.

The synthesis of the haptens started from kresoxim-methyl itself (haptens KMe and KMe) or from an aryl system that already incorporated the (*E*)-2-(methoxyimino)acetate moiety (haptens KMa, KMc, and KMo). In all cases, the alkyl C-6 carboxylated spacer arm was introduced in the form of *tert*-butyl ester, either through a Sonogashira cross-coupling reaction with the appropriate alkyne or by means of an *O*-alkylation reaction with the corresponding *tert*-butyl alkyl-bromoester. For all of the haptens, the synthesis was completed by acid hydrolysis of the *tert*-butyl ester moiety.

Fig. 3. Synthetic sequences for the preparation of haptens KMe and KMo.

As it is shown in Fig. 2, the syntheses of haptens KMa, KMe, and KMc followed a common strategy based on the initial preparation of an iodinated derivative of kresoxim-methyl. The rest of the steps to complete the synthesis of these haptens from the key iodinated intermediate (**3**, **7**, or **12**) implied palladium/cooper catalyzed Sonogashira cross-coupling reaction with the terminal triple bond of *tert*-butyl hex-5-ynoate (**4**) (to give aryl-alkynes **5**, **8**, or **13**), followed by hydrogenation of the triple bond under homogeneous conditions using Wilkinson's catalyst (to afford **6**, **9**, or **14**), and finally, acidic hydrolysis of the *tert*-butyl ester group to provide haptens KMa, KMe, and KMc in 61%, 73%, and 55% overall yield, respectively, from the corresponding starting material.

The synthesis of hapten KMe was readily accomplished in three steps (Fig. 3, left-hand side); *i.e.*, alkaline hydrolysis of methyl ester group of kresoxim-methyl to give carboxylic acid **15**, followed by *O*-alkylation of the carboxylate group with *tert*-butyl 6-bromohexanoate (**16**), and acid hydrolysis of the *tert*-butyl ester group. This straightforward sequence afforded hapten KMe in nearly 60% overall yield from the starting material.

Finally, for the synthesis of hapten KMo we made use of benzyl-bromide **19** (Fig. 3, right-hand side), prepared in high yield from oxime **18** in two successive steps, first *O*-alkylation of the oxime moiety with *tert*-butyl 6-bromohexanoate (**16**) followed by radical bromination of the benzylic position with NBS. The synthesis of hapten KMo was completed from **19** via *C*-alkylation reaction of the benzylic position with *o*-cresol followed by formic acid promoted hydrolysis of the *tert*-butyl ester group. The overall yield of the synthesis of hapten KMo from starting material **18** was about 68%.

Protein conjugate preparation

Bioconjugates to BSA, OVA, and HRP were prepared with each synthetic hapten using *N,N'*-disuccinimidyl carbonate as activating reagent. With this strategy, the active ester (NHS ester, see Fig. 1) of the hapten could be easily purified. Since pure activated haptens were employed for coupling, no unwanted secondary reactions occurred during conjugation; thus, the same chemical strategy could be followed both for immunogen and for assay antigen preparation. Immunizing conjugates showed hapten-to-protein molar ratios between 13 and 16, whereas molar ratios for OVA and HRP conjugates were between 3 and 5; meaning that around half of the available lysine ϵ -amine groups of BSA, one fourth of OVA, and all those of HRP most likely held hapten molecules.

Immune response and polyclonal antibody characterization

Two pAbs were generated from each immunogen, which were named after the immunizing hapten, and numbered. Every immunogen (BSA conjugates) was able to elicit a satisfactory immune response, with titers higher than 1/10000 as determined by direct ELISA. Evaluation of the affinity of antibodies raised by the different haptens was done by comparison of the IC_{50} values from assays with homologous conjugates, *i.e.*, those carrying the same hapten than the immunogen but coupled to HRP. The affinities of each pair of antisera that was generated from the same immunizing hapten were essentially equivalent (Table 1), perhaps with the only exception of the two pAbs produced from hapten KMa (*r*KMa#1 and *r*KMa#2), whose IC_{50} values differed 4-fold. The highest affinity to kresoxim-methyl was found with KMo-type antibodies (obtained from hapten KMo), followed by those triggered by hapten KMe. Contrarily, antibodies with higher IC_{50} values were produced from haptens containing the spacer arm at the b-ring (haptens KMe and KMc) or at the a-ring (hapten KMa). Among these three haptens, probably the most surprising and unexpected result was the poor performance of KMe-type antibodies because the position of the linker in hapten KMe should not theoretically cause steric hindrance, and minimum electronic or conformational modifications were involved.

Table 1 IC₅₀ (nM) values for kresoxim-methyl from assays using polyclonal antibodies.^a

pAb	Enzyme tracer				
	KMa	KMb	KMc	KMe	KMo
<i>rKMa</i> #1	28	– ^b	–	–	5
<i>rKMa</i> #2	117	–	–	–	25
<i>rKMb</i> #1	–	350	36	16	13
<i>rKMb</i> #2	–	300	19	9	–
<i>rKMc</i> #1	268	30	180	2	–
<i>rKMc</i> #2	76	1000	200	–	–
<i>rKMe</i> #1	23	12	4	11	5
<i>rKMe</i> #2	48	20	9	27	–
<i>rKMo</i> #1	–	–	–	4	3
<i>rKMo</i> #2	4	5	–	3	7

^a Values are the mean of three independent experiments. Homologous combinations are indicated in bold-type letter. ^b A_{max} was lower than 0.8 at the highest tested tracer concentration (300 ng mL⁻¹) and at the lowest assayed antiserum dilution (1/3000).

Fig. 4. Inhibition curves for the best pAb from each immunizing hapten as evaluated by homologous competitive ELISA. Values are the mean of three independent experiments.

Fig. 5. Standard inhibition curves for antibody *rKMc*#1 using the HRP-KMc homologous tracer (▲) and the HRP-KMe heterologous tracer (■). Values are the mean of three independent experiments.

The differences in kresoxim-methyl recognition exhibited by the produced pAbs as a function of the hapten derivatization site can be observed in Fig. 4. In previous studies with other strobilurins, functionalization through the toxophore moiety, particularly at the methoxy group, also resulted in optimum affinity responses.^{36–38} With the linker at such position, the characteristic aromatic rings of the molecule seem to be better exposed to the immune system. In contrast, the a-ring was revealed as a less adequate site for derivatization – particularly in *para* position from the (methoxyimino)acetate group – if high-affinity antibodies are sought.

It is well-known, particularly with pAbs, that changes in hapten structure can decrease the recognition towards the corresponding assay conjugate, thus enhancing the interaction of the antibody with the target molecule and therefore increasing the apparent affinity.^{9, 24, 25, 29, 30, 39, 40} Accordingly, kresoxim-methyl binding by the different antisera was tested with heterologous HRP conjugates (Table 1). The number of working combinations of immunoreagents was higher than expected, taking into account that linkers had been introduced at very different positions of the molecular framework in the immunizing and in the assay hapten. KMe-type antibodies were able to perfectly tolerate site-heterologies, whereas KMa-type antibodies just recognized the HRP-KMo heterologous tracer.

On the other hand, if adequate binding to the conjugate occurred, most heterologous combinations helped to considerably improve the antibody apparent affinity, mostly for those pAbs that had rendered high IC₅₀ values with the homologous conjugate. In fact, pAb *rKMc*#1 improved the IC₅₀ value 90-fold with the conjugate HRP-KMe (Fig. 5), and pAb *rKMb*#2 increased its apparent affinity 30-fold also with HRP-KMe. Nevertheless, most combinations with IC₅₀ values in the low nanomolar range still were obtained with antibodies raised by the best immunizing haptens, KMo and KMe.

Monoclonal antibody characterization

In order to generate high-affinity kresoxim-methyl binders, hybridomas were selected following a double screening competitive procedure. By this strategy, a collection of novel mAbs coming from mice immunized with each of the synthesized haptens was attained. Each antibody was named after the immunizing hapten, and numbered. All of the antibodies were comprised by κ light chains and were of the IgG₁ isotype, except mAbs KMa#26 and KMb#322 which were of the IgG_{2b} isotype.

Affinity-purified mAbs were characterized versus the homologous and all of the site-heterologous conjugates by checkerboard competitive direct ELISA. Half of the mAbs did not work satisfactorily, not even with the homologous enzyme tracer (Table 2). Such behavior upon mAb immobilization has been previously observed by several authors, and it is commonly ascribed to conformational changes of antibodies resulting from direct adsorption to hydrophobic surfaces.^{41–44} As a foremost result, working mAbs exhibited IC₅₀ values below 2 nM. Interestingly, HRP conjugates of haptens KMe and KMo – holding the linker at the (methoxyimino)acetate moiety – were bound by both KMe- and KMo-type antibodies, indicating a certain equivalency of these two adjacent derivatization sites. It is worthy to note that high-affinity mAbs were obtained from hapten KMb. This result contrasts with the poor affinity exhibited

by K**Mb**-type pAbs, and raises the question of whether information from rabbit antisera is a useful criterion in order to select optimum haptens for hybridoma technology. In addition, antibody K**Mb**#322 recognized all four heterologous tracers, yet similar IC₅₀ values were derived from all reagent combinations.

Table 2 IC₅₀ values (nM) for kresoxim-methyl from assays using monoclonal antibodies.^a

mAb	Enzyme tracer				
	K M <i>a</i>	K M <i>b</i>	K M <i>c</i>	K M <i>e</i>	K M <i>o</i>
K M <i>a</i> #26	– ^b	–	–	–	–
K M <i>a</i> #29	–	–	–	–	–
K M <i>b</i> #19	–	–	–	–	–
K M <i>b</i> #32	–	2.02	0.60	0.68	–
K M <i>b</i> #44	–	3.41	–	–	–
K M <i>b</i> #322	0.60	0.76	0.66	0.97	0.78
K M <i>c</i> #35	–	–	–	–	–
K M <i>e</i> #14	–	–	–	1.77	1.72
K M <i>e</i> #18	–	–	–	1.90	1.05
K M <i>e</i> #26	–	–	–	–	–
K M <i>e</i> #211	–	–	–	–	–
K M <i>o</i> #111	–	–	–	2.13	2.08
K M <i>o</i> #114	–	–	–	–	–
K M <i>o</i> #117	–	–	–	1.97	1.70

^a Values are the mean of three independent determinations and correspond to an inhibition curve with an A_{max} value between 0.8 and 1.5. Antibody concentration was either 300 or 1000 ng mL^{–1}. ^b A_{max} values were lower than 0.8 at 300 ng mL^{–1} tracer concentration and the highest coating concentration (mAb at 1 µg mL^{–1}).

To gain further insights into the kresoxim-methyl binding properties of the mAb collection, binders were also examined in solution by competitive ELISA with immobilized OVA–hapten conjugates. As shown in Table S1, general remarks were essentially identical; haptens K**M***o*, K**M***e* and K**M***b* were confirmed as the best mimics in order to generate high-affinity anti-kresoxim-methyl mAbs, whereas haptens K**M***a* and K**M***c* just elicited antibodies of moderate affinity. Moreover, the widest tolerance to site-heterologies was again observed with mAbs that had been triggered from hapten K**M***b*.

Conclusions

A differential response of the immune system to a battery of kresoxim-methyl regioisomeric haptens with the linker at a variety of sites was observed, thus proving a relationship between the structure of the immunizing hapten and the apparent affinity of derived antibodies. It was seen that derivatization at the *a*-ring of strobilurins triggered a moderate immune response in terms of affinity to kresoxim-methyl, as observed before for other compounds of this family. On the contrary, high-affinity polyclonal and monoclonal antibodies were obtained from immunogens with the linker at either end of the (methoxyimino)acetate moiety, which possess higher conformational freedom. Results from a hapten carrying the linker at the *para* position of the characteristic *o*-cresol ring (hapten K**M***b*) strongly differed between rabbit pAbs and mouse

mAbs. Despite of the drastic alteration introduced by site-heterologies, pAbs are complex enough to be able to bind such conjugates and to permit considerable sensitivity improvements, mainly with moderate-affinity antibodies. On the contrary, few mAbs could tolerate such severe changes in hapten structure, especially if the assay hapten held the linker at an opposite site with respect to the immunizing hapten.

Experimental

Chemicals and general procedures

tert-Butyl hex-5-ynoate and *tert*-butyl 6-bromohexanoate were prepared by esterification with isobutene of commercial hex-5-ynoic acid and 6-bromohexanoic acid, respectively, following a previously described procedure for the preparation of related *tert*-butyl esters.¹⁵ Other chemical reagents were purchased from regular sources and used without purification. All operations involving air-sensitive reagents were performed under an inert atmosphere of dry nitrogen or argon using syringe and cannula techniques, oven-dried glassware, and freshly distilled and dried solvents. Organic solvents were dried and distilled before use according to standard procedures.⁴⁵ Reactions were followed by thin-layer chromatography using 0.25 mm pre-coated silica gel plates and products were visualized by UV light (254 nm) and aqueous ceric ammonium molybdate solution or 50% (v/v) concentrated H₂SO₄ in water and then heating with a high-temperature air dryer. Unless otherwise stated, reaction mixtures were worked up by addition of water, and extraction with the appropriate solvent, the organic layer being washed with water and/or brine and dried using anhydrous magnesium or sodium sulfate. Evaporation of the solvent was performed under reduced pressure using a rotary evaporator. Synthesized compounds were purified by flash chromatography with a silica gel column (Merck 60, 230–400 mesh). Melting points and spectra were recorded as in previous studies [8].³⁸ The most stable conformation of kresoxim-methyl was calculated with CAChe WorkSystem Pro software, version 7.5.0.85 (Fujitsu Ltd, Tokyo, Japan).

Kresoxim-methyl (methyl (*E*)-methoxyimino[*α*-(*o*-tolylxy)-*o*-tolyl]acetate, CAS Registry No. 143390-89-0, MW 313.35 g mol^{–1}), Pestanal grade, was purchased from Fluka/Riedel-de-Haën (Seelze, Germany). Horseradish peroxidase (HRP), ovalbumin (OVA), *N,N*-disuccinimidyl carbonate (DSC), Freund's adjuvants, and *o*-phenylenediamine were purchased from Sigma/Aldrich (Madrid, Spain). Rabbit anti-mouse immunoglobulins polyclonal antibody–peroxidase conjugate (RAM–HRP) was from Dako (Glostrup, Denmark). Bovine serum albumin (BSA) fraction V and Hybridoma Fusion and Cloning Supplement (HFCS) were obtained from Roche Applied Science (Mannheim, Germany). P3-X63-Ag8.653 mouse plasmacytoma cell line was acquired from the European Collection of Cell Cultures (Wiltshire, UK). Polyethylene glycol Hybri-Max (PEG 1500), cell culture media (high-glucose Dulbecco's Modified Eagle's Medium, DMEM), non-essential amino acid solution, 200 mM alanyl-glutamine solution, fetal bovine serum (FBS), and red blood cell lysing buffer were bought to Sigma/Aldrich (Madrid, Spain). Gentamicine, hipoxanthine–aminopterin–thymidine (HAT), and hipoxanthine–thymidine (HT) solutions were obtained from Gibco BRL (Paisley,

Scotland). Culture plastic ware and Costar 96-well flat-bottom high-binding polystyrene ELISA plates were from Corning (Corning, NY, USA). Sephadex G-25 HiTrap Desalting columns and Sepharose HiTrap Protein G HP columns from GE-Healthcare (Uppsala, Sweden) were used for conjugate and for antibody purification, respectively, in the ÄKTA workstation. Ultraviolet-visible spectra and ELISA absorbances were read with a PowerWave HT from BioTek Instruments (Winooski, VT, USA). Microwells were washed with an ELx405 microplate washer also from BioTek Instruments. The immunoglobulin isotype was determined using the Mouse MonoAb-ID kit from Invitrogen (Carlsbad, CA, USA).

Composition, concentration, and pH of the employed buffers and solutions were as follows: i) CB, 50 mM sodium carbonate–bicarbonate buffer, pH 9.6; ii) PB, 100 mM sodium phosphate buffer, pH 7.4; iii) PBS, 10 mM sodium phosphate buffer, pH 7.4, with 140 mM NaCl; iv) PBST, PBS containing 0.05% (v/v) Tween 20; v) washing solution, 150 mM NaCl containing 0.05% (v/v) Tween 20; and vi) substrate solution, 0.012% (v/v) H₂O₂ and 2 mg mL^{−1} *o*-phenylenediamine in 25 mM sodium citrate and 62 mM sodium phosphate buffer, pH 5.4.

Syntheses of haptens

Five functionalized regioisomeric haptens of kresoxim-methyl with a C-6 hydrocarbon spacer arm located at selected sites of the molecular skeleton were synthesized (Fig. 1). Experimental details of their preparation are given below, while a detailed description of full spectroscopic data of all synthetic intermediates is provided in the Supplementary Data file (Fig. S1 to S5).

Synthesis of hapten KMa (Fig. 2)

Synthesis of (E)-methyl 2-(4-iodo-2-((o-tolyloxy)methyl)phenyl)-2-(methoxyimino)acetate (3).

A mixture of benzyl bromide **1** (112 mg, 0.272 mmol), prepared in three steps from 1-iodo-3-methylbenzene (see the Supplementary Data file), *o*-cresol (**2**, 28 μ L, 0.272 mmol), and anhydrous Cs₂CO₃ (133 mg, 0.408 mmol) in dry *N,N*-dimethylformamide (DMF) (1.3 mL) was stirred under nitrogen for 6 h, poured into water, and worked up using EtOAc. Chromatography of the residue left after evaporation of the solvent, using hexane–Et₂O (from 9:1 to 8:2) as eluent, afforded benzyl ether **3** (107.5 mg, 90%) as an oil.

Synthesis of (E)-tert-butyl 6-(4-(2-methoxy-1-(methoxyimino)-2-oxoethyl)-3-((o-tolyloxy)methyl)phenyl)hex-5-ynoate (5).

A solution of *tert*-butyl hex-5-ynoate (**4**, 155 mg, 0.920 mmol) in anhydrous degassed DMF (1.0 mL) was added to a mixture of aryl iodide **3** (101 mg, 0.230 mmol), CuI (1.3 mg, 0.007 mmol, 3% mol) and (Ph₃P)₂PdCl₂ (4.8 mg, 0.007 mmol, 3% mol) under nitrogen atmosphere. After the addition anhydrous Et₃N (0.5 mL), the reaction mixture, initially yellow that turned deep-red after a few minutes, was stirred at room temperature for 6 h, then poured into water, and worked up in the usual manner using EtOAc to extract. Work up as usual and chromatography of the crude product with hexane–Et₂O (7:3) as eluent afforded alkyne **5** (94.7 mg, 86%) as a colorless oil.

Synthesis of (E)-tert-butyl 6-(4-(2-methoxy-1-(methoxyimino)-2-oxoethyl)-3-((o-tolyloxy)methyl)phenyl)hexanoate (6).

A mixture of alkyne **5** (56.3 mg, 0.117) and Rh(PPh₃)₃Cl (3.3 mg, 0.003 mmol) in anhydrous tetrahydrofuran (THF; 1 mL) was

stirred under an atmosphere of hydrogen at 4 atm during 16 h. The reaction mixture was concentrated to dryness and the obtained residue was purified by chromatography, using hexane–EtOAc (9:1) as eluent, to give the title compound **6** (48.2 mg, 85%) as a brownish oil.

Synthesis of (E)-6-(4-(2-methoxy-1-(methoxyimino)-2-oxoethyl)-3-((o-tolyloxy)methyl)phenyl)hexanoic acid (hapten KMa).

A solution of *tert*-butyl ester **6** (48.2 mg, 0.099 mmol) in formic acid (2 mL) was stirred at room temperature under nitrogen for 3 h. Then, the reaction mixture was diluted with benzene and washed with cold water until pH 6–7, and dried over anhydrous Na₂SO₄. Chromatographic purification of the residue left after evaporation of the solvent, using CHCl₃ as eluent, afforded hapten KMa (39.3 mg, 93%) as colorless oil.

Synthesis of hapten KMb (Fig. 2)

Synthesis of (E)-methyl 2-(2-((4-iodo-2-methylphenoxy)methyl)phenyl)-2-(methoxyimino)acetate (7).

A 1.53 M solution of ICl in CH₂Cl₂ (4.5 mL, 6.885 mmol) was added drop wise to a solution of kresoxim-methyl (537.5 mg, 1.720 mmol) in AcOH (3 mL) at room temperature. The resulting mixture was stirred during 5 h, then poured into water and extracted with EtOAc. The combined organic layers were washed successively with a 10% aqueous solution of sodium thiosulfate, water and brine, dried over anhydrous Na₂SO₄ and concentrated, and the obtained residue was purified by chromatography, using hexane–EtOAc (8:2) as eluent, to give the aryl iodide **7** (718.4 mg, 95%) as a white solid. Mp. 124–125 °C (crystallized from hexane–benzene).

Synthesis of (E)-tert-butyl 6-(4-((2-(2-methoxy-1-(methoxyimino)-2-oxoethyl)benzyl)oxy)-3-methylphenyl)hex-5-ynoate (8).

The synthesis of this compound was performed following the same procedure reported for compound **5**, starting from aryl iodide **7** (529.8 mg, 1.21 mmol), alkyne **4** (811 mg, 4.84 mmol), CuI (6.9 mg, 0.040 mmol), Pd(PPh₃)₂Cl₂ (25.5 mg, 0.040 mmol), DMF (3.9 mL), and Et₃N (2.9 mL), and 3 h of reaction time. The crude product was purified by chromatography, using hexane–EtOAc (from 9:1 to 8:2) as eluent, to give alkyne **8** (545.8 mg, 95%) as a yellowish oil.

Synthesis of (E)-tert-butyl 6-(4-((2-(2-methoxy-1-(methoxyimino)-2-oxoethyl)benzyl)oxy)-3-methylphenyl)hexanoate (9).

Alkyne **8** (499.4 mg, 1.041 mmol) was hydrogenated as described above for the preparation of **6** using Rh(PPh₃)₃Cl (28.9 mg, 0.031 mmol) and THF (9.5 mL). Purification of the crude product by chromatography with hexane–EtOAc (8:2) as eluent gave title compound **9** (455.8 mg, 90%) as a semisolid.

Synthesis of (E)-6-(4-((2-(2-methoxy-1-(methoxyimino)-2-oxoethyl)benzyl)oxy)-3-methylphenyl)hexanoic acid (hapten KMb).

Hydrolysis of the *tert*-butyl ester moiety of **9** (159 mg, 0.329 mmol) with formic acid (6.8 mL) under the same conditions reported above for **6**, followed by purification by chromatography using CHCl₃ as eluent, afforded hapten KMb (127.8 mg, 91%) as a slightly colored oil.

Synthesis of hapten KMc (Fig. 2)

Synthesis of (E)-methyl 2-(2-((2-iodophenoxy)methyl)phenyl)-2-(methoxyimino)acetate (12).

This compound was prepared basically according to the procedure already described for compound **6** starting from benzyl bromide **10** (74.2 mg, 0.259 mmol),⁴⁶ 2-iodophenol (**11**, 30 μ L, 0.259 mmol), and Cs₂CO₃ (125 mg, 0.388 mmol) in DMF (1.5

mL), and 4 h of reaction time. The crude product was purified by chromatography, using hexane–Et₂O (7:3) as eluent, to give title compound **12** (72.6 mg, 90%) as a white solid. Mp. 87–89 °C (crystallized from hexane–benzene).

Synthesis of (E)-tert-butyl 6-(2-((2-methoxy-1-(methoxyimino)-2-oxoethyl)benzyl)oxy)phenyl hex-5-ynoate (13**).**

The synthesis of this compound was performed following the same procedure that was described above for compound **5**, starting from aryl iodide **12** (78.0 mg, 0.239 mmol), alkyne **4** (48.0 mg, 0.287 mmol), CuI (1.4 mg, 0.007 mmol), Pd(PPh₃)₂Cl₂ (5.0 mg, 0.007 mmol), DMF (1.0 mL), and Et₃N (0.5 mL), and 2 h of reaction time. The crude product was purified by chromatography, using hexane–Et₂O (8:2) as eluent, to give **13** (65.6 mg, 75%) as a colorless oil.

Synthesis of (E)-tert-butyl 6-(2-((2-methoxy-1-(methoxyimino)-2-oxoethyl)benzyl)oxy)phenyl hexanoate (14**).**

Alkyne **13** (65.6 mg, 0.180 mmol) was hydrogenated as described above for the preparation of **6** using Rh(PPh₃)₃Cl (5.0 mg, 0.005 mmol) and THF (1.6 mL). Purification of the crude product by chromatography with hexane–Et₂O (from 9:1 to 8:2) as eluent gave title compound **14** (60.8 mg, 91 %) as a colorless oil.

Synthesis of (E)-6-(2-((2-methoxy-1-(methoxyimino)-2-oxoethyl)benzyl)oxy)phenylhexanoic acid (hapten **KMc).**

Hydrolysis of the *tert*-butyl ester group of **14** (60.8 mg, 0.165 mmol) with formic acid (3.4 mL) under the same conditions reported above for **6** afforded, after chromatographic purification with CHCl₃ as eluent, hapten KMc (46.5 mg, 90%) as a white solid. Mp. 86–89 °C (crystallized from hexane).

Synthesis of hapten KMe (Fig. 3)

Synthesis of (E)-tert-butyl 6-(2-(methoxyimino)-2-((*o*-tolyl)oxy)methyl)phenylacetoxyl hexanoate (17**).**

tert-Butyl 6-bromohexanoate (**16**, 100.6 mg, 0.401 mmol) was added to a mixture of acid **15** (100 mg, 0.334 mmol), obtained by basic hydrolysis of the methyl ester moiety of kresoxim-methyl following the procedure previously described,¹² and anhydrous K₂CO₃ (54.4 mg, 0.401 mmol) in dry DMF (13 mL) under nitrogen at room temperature. The resulting mixture was stirred for 24 h, then poured into water, and worked up in the usual manner extracting with EtOAc. Purification of the crude product by chromatography, using hexane–Et₂O (from 8:2 to 7:3) as eluent, afforded title compound **17** (110 mg, 70%) as a colorless oil.

Synthesis of (E)-6-(2-(methoxyimino)-2-((*o*-tolyl)oxy)methyl)phenylacetoxylhexanoic acid (hapten **KMe).**

Hydrolysis of the *tert*-butyl ester group of **17** (36.9 mg, 0.078 mmol) with formic acid (1.6 mL) under the same conditions reported above for **6** afforded, after chromatographic purification with CHCl₃ as eluent, hapten KMe (30.6 mg, 93%) as a colorless oil.

Synthesis of hapten KMo (Fig. 3)

Preparation of (E)-tert-butyl 6-(((1-(2-(bromomethyl)phenyl)-2-methoxy-2-oxoethylidene)amino)oxy)hexanoate (19**).**

A mixture of oxime **18** (synthesized following the procedure described by Li et al.)^{47, 48} (90 mg, 0.466 mmol), *tert*-butyl 6-bromohexanoate (**16**, 117 mg, 0.466 mmol), and anhydrous Cs₂CO₃ (228 mg, 0.699 mmol) in dry DMF (1.7 mL) was stirred under nitrogen for 4 h, poured into water, and worked up as usual using EtOAc to extract. Chromatography of the crude product, using hexane–EtOAc (9:1) as eluent, afforded the corresponding intermediate *O*-alkylated oxime (141 mg, 83%) as a colorless oil.

A solution of the above obtained oxime (123 mg, 0.339 mmol), NBS (60.3 mg, 0.339 mmol), and AIBN (3.3 mg, 0.020 mmol) in anhydrous benzene (1.5 mL) was degassed by nitrogen bubbling and ultrasound for 15 min and then heated at reflux overnight.

The reaction mixture was allowed to cool at room temperature, diluted with EtOAc, and worked up as usual to afford the crude product as a pale brown oil. Purification by column chromatography, using hexane–Et₂O (from 9:1 to 8:2) as eluent, gave benzyl bromide **19** (137.8 mg, 92%; 76% overall yield from **18**) as a brownish oil.

Synthesis of (E)-tert-butyl 6-(((2-methoxy-2-oxo-1-(2-((*o*-tolyl)oxy)methyl)phenyl)ethylidene)amino)oxy)hexanoate (20**).**

This compound was prepared following the procedure described above for compound **3** but starting from benzyl bromide **19** (54.7 mg, 0.124 mmol), *o*-cresol (12.8 μL, 0.124 mmol), and Cs₂CO₃ (60.6 mg, 0.186 mmol) in DMF (522 μL). The crude product was purified by chromatography, using hexane–Et₂O (9:12) as eluent, to give title compound as a colorless oil (57.6 mg, 99%).

Synthesis of (E)-6-(((2-methoxy-2-oxo-1-(2-((*o*-tolyl)oxy)methyl)phenyl)ethylidene)amino)oxy) hexanoic acid (hapten **KMo).**

Hydrolysis of the *tert*-butyl ester moiety of **20** (115.2 mg, 0.246 mmol) with formic acid (5.0 mL) was carried out under the same conditions reported above for **6**, and after chromatographic purification with CHCl₃ as eluent, hapten KMo (91.4 mg, 90%) was obtained as a colorless oil.

Preparation of hapten active esters

The synthesized haptens were activated by formation of the corresponding *N*-hydroxysuccinimidyl ester (NHS ester) using DSC. Anhydrous Et₃N (54 μL, 0.38 mmol, 3.8 equiv) was added to a solution of the hapten (0.1 mmol) and DSC (26 mg, 0.13 mmol, 1.3 equiv) in dry acetonitrile (1 mL) under nitrogen at 0 °C. The reaction mixture was stirred at room temperature overnight, then diluted with CHCl₃, washed with a 10% aqueous solution of NaHCO₃ and brine, and dried over anhydrous Na₂SO₄. Chromatographic purification of the residue that was left after evaporation of the solvent, using CHCl₃ as eluent, afforded the NHS esters in good yield (70–99%) and high purity, as shown by ¹H NMR spectroscopy (copies of original spectra are included in the Supplementary Data file).

KMa-NHS ester: (88% yield). ¹H NMR (CDCl₃, 300 MHz) δ 7.37 (1H, br s, H-2'), 7.20 (1H, dd, *J* = 7.8, 1.5 Hz, H-6'), 7.14–7.07 (3H, m, H-5', H-3'' and H-5''), 6.85 (1H, br dd, *J* = 7.5, 7.5 Hz, H-4''), 6.78 (1H, br d, *J* = 8.1 Hz, H-6''), 4.93 (2H, s, OCH₂), 4.02 (3H, s, NOCH₃), 3.81 (3H, s, CO₂CH₃), 2.82 (4H, br s, OCCH₂CH₂CO), 2.67 (2H, t, *J* = 7.5 Hz, H-6), 2.59 (2H, t, *J* = 7.5 Hz, H-2), 2.24 (3H, s, CH₃), 1.78 and 1.69 (2H each, two partially overlapped quint, *J* = 7.5 Hz, H-5 and H-3), 1.45 (2H, m, H-4).

KMb-NHS ester: (70% yield). ¹H NMR (C₆D₆, 300 MHz) 7.45 (1H, dd, *J* = 7.4, 1.3 Hz, H-3''), 7.16 (1H, signal hidden by solvent peak, H-6''), 7.08 (1H, ddd, *J* = 7.4, 7.4, 1.7 Hz, H-4''), 7.02 (1H, ddd, *J* = 7.4, 7.4, 1.3 Hz, H-5''), 6.89 (1H, br s, H-2''), 6.83 (1H, dd, *J* = 8.3, 1.9 Hz, H-6'), 6.72 (1H, d, *J* = 8.3 Hz, H-5'), 5.02 (2H, s, OCH₂), 3.61 (3H, s, NOCH₃), 3.36 (3H, s, CO₂CH₃), 2.37 (5H, s and t overlapped, CH₃ and H-6), 2.11 (2H, t, *J* = 7.4 Hz, H-2), 1.7–1.5 (4H, m, OCCH₂CH₂CO), 1.5–1.3 (4H, two partially overlapped quint, *J* = 7.7 Hz, H-5 and H-3),

1.12 (2H, m, H-4).

KMc-NHS ester: (98% yield). ^1H NMR (CDCl_3 , 300 MHz) δ 7.58 (1H, dd, $J = 7.6, 1.5$ Hz, H-3''), 7.45 (1H, ddd, $J = 7.6, 7.6, 1.5$ Hz, H-4''), 7.38 (1H, ddd, $J = 7.6, 7.6, 1.5$ Hz, H-5''), 7.19 (1H, dd, $J = 7.6, 1.5$ Hz, H-6''), 6.88 (1H, ddd, $J = 7.5, 7.5, 1.2$ Hz, H-5'), 6.77 (1H, d, $J = 8.1$ Hz, H-3'), 4.95 (2H, s, OCH_2), 4.04 (3H, s, NOCH_3), 3.84 (3H, s, CO_2CH_3), 2.83 (4H, br s, $\text{OCCH}_2\text{CH}_2\text{CO}$), 2.66 (2H, t, $J = 7.5$ Hz, H-6), 2.57 (2H, t, $J = 7.5$ Hz, H-2), 1.77 (2H, quint, $J = 7.5$ Hz, H-5), 1.64 (2H, quint, $J = 7.5$ Hz, H-3), 1.47 (2H, m, H-4).

KMe-NHS ester: (98% yield). ^1H NMR (CDCl_3 , 300 MHz) δ 7.57 (1H, br d, $J = 7.5$ Hz, H-6'), 7.44 (1H, ddd, $J = 7.5, 7.5, 1.5$ Hz, H-5'), 7.38 (1H, ddd, $J = 7.5, 7.5, 1.4$ Hz, H-4'), 7.21 (1H, dd, $J = 7.5, 1.5$ Hz, H-3'), 7.15–1.07 (2H, m, H-3'' and H-5''), 6.86 (1H, ddd, $J = 7.5, 7.5, 1.0$ Hz, H-4''), 6.77 (1H, br d, $J = 8.0$ Hz, H-6''), 4.95 (2H, s, OCH_2), 4.21 (2H, t, $J = 6.8$ Hz, H-6), 4.03 (3H, s, NOCH_3), 2.81 (4H, br s, $\text{OCCH}_2\text{CH}_2\text{CO}$), 2.52 (2H, t, $J = 7.5$ Hz, H-2), 2.25 (3H, s, CH_3), 1.66 (4H, m, H-3 and H-5), 1.37 (2H, m, H-4).

KMo-NHS ester: (98% yield). ^1H NMR (CDCl_3 , 300 MHz) δ 7.57 (1H, br d, $J = 7.5$ Hz, H-6'), 7.44 (1H, ddd, $J = 7.5, 7.5, 1.5$ Hz, H-5'), 7.38 (1H, ddd, $J = 7.5, 7.5, 1.5$ Hz, H-4'), 7.19 (1H, dd, $J = 7.5, 1.2$ Hz, H-3'), 7.11 (2H, m, H-3'' and H-5''), 6.85 (1H, ddd, $J = 7.2, 7.2, 0.9$ Hz, H-4''), 6.76 (1H, br d, $J = 7.8$ Hz, H-6''), 4.95 (2H, s, OCH_2), 4.24 (2H, t, $J = 6.9$ Hz, H-6), 3.81 (3H, s, CO_2CH_3), 2.81 (4H, br s, $\text{COCH}_2\text{CH}_2\text{CO}$), 2.50 (2H, t, $J = 7.5$ Hz, H-2), 2.24 (3H, s, CH_3), 1.66 (4H, two overlapped quint, $J = 7.8$ Hz, H-3 and H-5), 1.37 (2H, m, H-4).

Preparation of bioconjugates

For immunogen preparation, 100 μL of a 50 mM purified activated hapten (NHS ester) solution in DMF was added to a 1 mL BSA solution (15 mg mL^{-1}) in CB. OVA conjugates were prepared by addition of 100 μL of a 30 mM purified activated hapten solution in DMF over 2 mL of protein solution (15 mg mL^{-1}) in CB. Likewise, enzyme tracers were prepared by adding 100 μL of a 10 mM purified hapten-NHS ester solution in DMF over a 1 mL HRP solution (2.2 mg mL^{-1}) in CB. Mixtures were incubated 4 h at room temperature in amber glass vials and conjugates were purified by size-exclusion chromatography using PB as eluent. Hapten-to-protein molar ratios were calculated from absorbance values of the carrier before and after conjugation. BSA and OVA conjugates were stored frozen whereas HRP tracers were kept at 4 $^\circ\text{C}$ with 0.5% (w/v) BSA and 0.01% (w/v) thimerosal.

Generation of polyclonal antibodies

Animal manipulation was performed in compliance with the laws and guidelines of the Spanish Ministry of Agriculture, Fisheries, and Food and according to European Directive 2010/63 concerning protection of animals used for scientific purposes. Two antisera were generated with each immunogen from two 2 kg female New Zealand white rabbits, which were subcutaneously immunized with 0.3 mg of BSA-hapten conjugate in 1 mL of a 1:1 mixture of PB and complete Freund's adjuvant. At 21-day intervals, animals were boosted with a 1:1 emulsion between incomplete Freund's adjuvant and a PB solution containing 0.3 mg of the same immunogen. Ten days after the fourth injection, whole blood was collected from the ear

vein of the rabbits and by intracardiac puncture. Blood samples were allowed to coagulate overnight at 4 $^\circ\text{C}$; then, the serum was separated by centrifugation and immunoglobulins were precipitated with 1 volume of saturated ammonium sulfate solution. This procedure was repeated and the precipitates were stored at 4 $^\circ\text{C}$.

Generation of monoclonal antibodies

A set of four BALB/c female mice (8–10 weeks old) was immunized by intraperitoneal injections with each BSA-hapten conjugate following standard protocols.⁴⁹ Briefly, the immunization schedule consisted of four injections given at three week intervals. Each mouse received 100 μg of conjugate in 200 μL of a 1:1 mixture of PBS and Freund's adjuvant (complete for the first dose and incomplete for the second and third boosts). The fourth injection was done without adjuvant four days before mouse splenocytes were obtained and fused with myelomas. Cell fusion was performed at a 4:1 ratio using 1 mL of PEG 1500 as the fusing agent. Fused cells were distributed in 96-well culture plates at 1.5×10^5 lymphocytes per well in 100 μL of DMEM containing 15% (v/v) FBS. Twenty-four hours after plating, 100 μL of selection medium (DMEM supplemented with HAT) with 20% (v/v) FBS and 1% (v/v) HFCS was added to each well.

Twelve days after cell fusion, hybridoma culture supernatants were assayed following a sequential double-screening process. First, cell cultures were screened by differential indirect competitive ELISA with plates coated at 1.0 mg mL^{-1} OVA-hapten conjugate, as described by Abad et al.⁵⁰ Briefly, each culture supernatant was analyzed in parallel with and without competitor (100 nM kresoxim-methyl), and the obtained absorbances were compared. The ratio between those assay signals was used as the criterion for identification of the best clones that secreted antibodies recognizing not only the immobilized antigen (positive result) but also the target molecule; i.e., kresoxim-methyl (competitive result). In a second screening assay, culture supernatants from positive and competitive wells, and also from those wells that afforded saturated signals in the first screening experiment, were re-evaluated by checkerboard indirect competitive ELISA as described by Mercader et al.¹⁵ Serial dilutions of the supernatant were assayed in the presence of kresoxim-methyl at different concentrations (0, 10, and 100 nM), using ELISA plates that had been coated with the homologous OVA-hapten conjugate at 0.1 and 1.0 $\mu\text{g mL}^{-1}$. Selected hybridomas were cloned twice by limiting dilution in cloning medium (DMEM containing 20% (v/v) FBS and supplemented with HT and 1% (v/v) HFCS). Finally, stable antibody-producing clones were expanded and cryopreserved in liquid nitrogen. Antibodies were purified from late stationary-phase culture supernatants by salting out with ammonium sulfate and subsequent affinity chromatography, and stored at 4 $^\circ\text{C}$ as ammonium sulfate precipitates.

Competitive direct ELISAs

Immunoassays were performed using the competitive ELISA method with immobilized anti-kresoxim-methyl antibody. Briefly, plates were coated with 100 μL per well of antibody dilution in CB by overnight incubation at room temperature. After each incubation step, microwells were washed four times with washing solution. The competitive reaction was run by

addition of 50 μL per well of analyte in PBS plus 50 μL per well of HRP-hapten solution in PBST, and incubation during 1 h at room temperature. Signal was produced with 100 μL per well of freshly prepared enzyme substrate solution. After 10 min at room temperature, enzyme activity was stopped upon adding 100 μL per well of 2.5 M sulfuric acid. Absorbances were immediately read at 492 nm with a reference wavelength at 650 nm.

Standard curves and data analysis

Standard curves were set up from 10^{-2} $\mu\text{g L}^{-1}$ to 10^4 $\mu\text{g L}^{-1}$ by 10-fold serial dilution in PBS. A blank (no kresoxim-methyl) was also included. Experimental values were fitted to a four-parameter logistic equation using the SigmaPlot software package from SPSS Inc. (Chicago, IL, USA). Antibody affinity was estimated as the concentration of kresoxim-methyl at the midpoint of the fitted curve, typically corresponding to a 50% inhibition (IC_{50}) of the maximum absorbance (A_{max}) if the background signal approaches to zero. Polyclonal antibody titer was defined as the antiserum dilution that was required in order to achieve both an A_{max} around 1.0 and a minimum IC_{50} value in homologous assays.

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Limited amounts of the described immunoreagents are available upon request.

Notes and references

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[†] Electronic Supplementary Information (ESI) available: full characterization data of all the intermediate compounds in the synthesis of haptens and copies of ^1H NMR spectra. See DOI: 10.1039/b000000x/

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