Immunochemical rapid determination of quinoxifen, a priority hazardous pollutant

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ABSTRACT

In 2013, quinoxifen was included in the list of priority hazard pollutants of the European Water Framework Directive due to its toxicity to aquatic organisms. However, few analytical methods for the analysis of this fungicide have been reported and no rapid immunochemical methods have been published so far. In the present study, immunoreagents for quinoxifen analysis were generated for the first time and an enzyme-linked immunosorbent assay was developed. Two carboxylated derivatives of quinoxifen were designed on the basis of the minimum energy conformation of the target compound. Active esters of those novel compounds were prepared using *N,N'*-disuccinimidyl carbonate, and purified for covalent coupling to proteins. Matrix-assisted laser desorption mass spectrometry of the prepared bioconjugates showed optimum hapten-to-protein molar ratios. Moreover, high-affinity antibodies specific of quinoxifen were raised. As proof of concept, an immunoassay was evaluated using a heterologous conjugate, which afforded sensitivity values in the low nanomolar range. Moreover, excellent recoveries and coefficients of variation were obtained from the analysis of environmental water samples fortified with quinoxifen. A limit of quantification of 60 µg/L was determined. The prepared bioconjugates and antibodies could be valuable immunoreagents for the development of a variety of rapid immunosensors for quinoxifen determination in environmental samples.

KEYWORDS

Water pollutant; Hapten; Bioconjugate; Antibody; Immunochemical methods.

1. Introduction

Quinoxifen (Fig. 1) is the only fungicide listed in Annex I of the European Directive 2013/39/EU. This document amends the Water Framework Directive 2000/60/EC for priority hazardous pollutants that pose a significant risk to the aquatic environment, and it sets EU Environmental Quality Standards for those substances in water, sediment, and/or biota (SCHER, 2011; EU Directive 2013/39). Quinoxifen shows low toxicity to humans; however, the available evidence indicates that it may be considered a highly persistent substance in soil and water, and it also fulfills the very bioaccumulative criteria in fish (FAO, 2006; EFSA, 2018). The lethal toxicity threshold for crustaceans is commonly in the range of 3.0 to 91 µg/L. Particularly, feeding *Gammarus* sp. with quinoxifen was shown to be highly toxic ($EC_{50} = 23.8$ µg/L) (Maltby et al., 2002; Zubrod et al., 2014). Consequently, the European Commission considered monitoring of this compound to be decidedly necessary in order to protect aquatic and terrestrial organisms. Studies about the fate and behavior of quinoxifen in the environment can be scarcely found, probably due to the high sampling and analysis costs (Liess and Von der Ohe, 2005; Merli et al., 2010; Ferri et al., 2017), and only few analytical methods based on gas or liquid chromatography have been published (Pareja et al., 2011; Loos, 2012).

Nowadays, immunoassays are well-established techniques for the analysis of pollutants in environmental samples with screening, confirmatory, or quantification purposes. Particularly, the competitive enzyme-linked immunosorbent assay (cELISA) is commonly used for the determination of water contaminants as a rapid, economic, portable, and user-friendly analytical approach (Krall et al., 2017). The successful generation of high-affinity and specific antibodies to a small organic molecule, such as quinoxifen, is greatly dependent upon a proper design of immunizing and assay haptens. Due to the low molecular weight of this compound, covalent protein conjugates are required for immunization and competitive assay development. Thus, the preparation of synthetic derivatives of the target molecule (haptens) is usually mandatory in order to introduce functional chemical groups enabling protein coupling.

The conformation and electronic properties of the synthesized molecule should resemble as closely as possible those of the target ligand (Zhang and Wang, 2009). Moreover, a linear spacer arm of 3 to 6 atom length is preferentially introduced at a distal position from the most relevant moieties (Landsteiner, 1962; Mercader et al., 2008).

To our knowledge, no previous studies reporting the development of immunoassays specific of quinoxifen have been published so far. Therefore, the aim of the present study was to provide a complementary immunoanalytical method for the rapid and sensitive determination of this environmental pollutant. Consequently, quinoxifen derivatives were designed, conjugates were prepared, and antibodies were generated. As a preliminary approach, two cELISA formats were studied in order to select the best immunoreagents.

2. Materials and methods

2.1. Reagents and equipment

Quinoxifen [5,7-dichloro-4-quinolyl 4-fluorophenyl ether] (CAS registry number 124495-18-7, Mw 308.13) analytical standard was kindly provided by Dow AgroSciences (Indianapolis, IN). Detailed information regarding reagents, equipment, and general experimental details are given as Supplementary Material.

2.2. Hapten synthesis, activation, and conjugation

The synthetic strategy for the preparation of the two quinoxifen haptens is schematized in Fig. 1. Compounds used in this study present minor safety concerns; however, it is advisable to perform the synthetic work in a well-ventilated fume hood. Preparation of intermediate **1** was accomplished following an adaptation of the procedure previously described by Takamura et al., 2001 (Fig. S1). Detailed synthetic procedures and characterization data of all of the intermediates and haptens are provided as Supplementary Material. Activation of the carboxyl group of haptens QXt and QXf was accomplished by reaction with *N,N'*-disuccinimidyl carbonate and Et₃N (Fig. 1), as previously reported by our group (López-Moreno et al., 2013).

The **resulting** crude NHS esters had a purity greater than 95%, as indicated by ^1H NMR analysis, so they were used without additional purification for the preparation of the protein conjugates. Both haptens were covalently coupled to the free amine groups of bovine serum albumin (BSA), ovalbumin (OVA), and horseradish peroxidase (HRP), and conjugates were purified by size exclusion chromatography ([Supplementary Material](#)). The number of **hapten** molecules was determined by MALDI-TOF/MS. BSA and OVA conjugates were stored at $-20\text{ }^{\circ}\text{C}$ and HRP conjugates were kept concentrated at $4\text{ }^{\circ}\text{C}$.

2.3. *Antibody generation*

Two antisera were raised with each immunogen from New Zealand white rabbits. Animal manipulation was carried out in compliance with Spanish laws and guidelines (RD1201/2005 and law 32/2007) and according to the European Directive 2010/63EU concerning the protection of animals used for scientific purposes. The immunization procedure is described in the [Supplementary Material file](#).

2.4. *Competitive ELISAs*

Immunoassays were carried out by the antibody-coated direct and the conjugate-coated indirect cELISA formats. Details about the experimental procedures can be found in the [Supplementary Material file](#). Eight-point quinoxifen standard curves, including a blank, were prepared in borosilicate glass vials by serial dilution in buffer from a quinoxifen solution in *N,N*-dimethylformamide. **Absorbance values in ELISA microtiter plates were read** at 492 nm, with a reference wavelength at 650 nm. The SigmaPlot software from SPSS Inc. (Chicago, IL) was used for fitting the experimental values to a four-parameter logistic equation. Assay sensitivity was defined as the concentration of quinoxifen at the inflexion point of the sigmoidal curve, typically corresponding to a 50% inhibition (IC_{50}) of the maximum signal (A_{max}).

3. Results and discussion

3.1. *Hapten design and synthesis*

From a structural point of view, quinoxifen is a relatively rigid molecule with only two possible rotating bonds, *i.e.*, the C–O single bonds joining the ethereal oxygen atom with both the quinoline and phenyl rings. The molecule is not flat; in the lowest energy conformations, the phenyl ring adopts a pseudo-orthogonal disposition with respect to the plane of the quinoline ring (Fig. 1). This geometry of the quinoxifen molecule suggest that the C-4 position of the phenyl ring could probably be the most adequate position for its conjugation to the immunogenic carrier protein. Replacement of the fluorine atom by a carboxylated hydrocarbon chain should allow the better exposure of the most highly immunogenic elements of quinoxifen during the immune response. Furthermore, the incorporation of the hydrocarbon spacer arm at this position does not imply relevant structural or electronic changes and it does not impede the quinoxifen framework to cover the same conformational space as the parent molecule.

Based on the above considerations, we have designed two haptens of quinoxifen, namely QXf and QXt (Fig. 1). The former incorporates a linear saturated hydrocarbon chain with a terminal carboxylate group for conjugation to the proteins, while the second has a carboxylated spacer arm of the same length with a triple bond that joins it to the C-4 position of the phenyl ring. The presence of the linear, rigid, triple carbon-carbon bond in the hydrocarbon chain results in a less flexible spacer arm. In a previous study, we evaluated, although with a much simpler molecule (Esteve-Turrillas et al., 2015), the influence of this type of spacer arm over the immune response; however, the results were not clearly conclusive although they suggested that a spacer arm with a triple carbon–carbon bond could favor the production of antibodies with superior affinity.

The syntheses of the above mentioned haptens was undertaken from an iodinate analogue of quinoxifen – aryl iodide **3** – readily available from 4,5,7-trichlorquinoline (**1**), and are based on the use of a palladium catalyzed Sonogashira cross-coupling reaction for the incorporation of the carboxylated hydrocarbon chain at the C-4 position of the phenyl ring (Supplementary

Material). Pure haptens QXt and QXf were obtained from **1**, as trifluoroacetate quinolinium salts, through a sequence of **three and four steps and** with an overall yield of 58% and 55%, respectively (Fig. 1). **As in previous studies, the Sonogashira cross-coupling reaction was demonstrated to be an effective strategy to prepare functionalized haptens with the spacer arm at a halogenated aromatic ring (Parra et al., 2011; López-Moreno et al., 2014; Esteve-Turrillas et al., 2015).**

3.2. Bioconjugate preparation

Carboxylated haptens were activated by the formation of the corresponding *N*-hydroxysuccinimidyl ester using *N,N*-disuccinimidyl carbonate and Et₃N in anhydrous acetonitrile (Fig. 1). The activation reaction proceeded from the trifluoroacetate quinolinium salts of the haptens, leading to **the highly pure** *N*-hydroxysuccinimidyl esters QXf-NHS (35.7 mg from 40.8 mg of hapten QXf, 90.4% yield) and QXt-NHS (27.9 mg from 25.9 mg of hapten QXt, 91.7% yield).

Since pure activated haptens were employed, the conjugates were readily accomplished with high yields. **Moreover, a single coupling procedure was followed for both immunizing and assay conjugates in comparison to the classic one-pot coupling strategy in which two different activation methods are recommendable.** The obtained hapten-to-protein molar ratios for hapten QXt were 17.3, 4.1, and 1.7 for BSA, OVA, and HRP conjugates, respectively. Those of hapten QXf were 14.5, 3.1, and 1.3 for BSA, OVA, and HRP conjugates, respectively. These values were within the expected optimum range for immunizing and assay conjugates. MALDI spectra of the prepared conjugates can be found **as Supplementary Material (Fig. S2, S3 and S4).**

3.3. Antibody characterization

Two antibodies were produced from each of the two BSA conjugates carrying the synthetic quinoxifen haptens. Antibodies QXt-1 and QXt-2 were generated with the conjugate of hapten

QXt, and antibodies QXf-1 and QXf-2 were raised with the conjugate BSA–QXf. The immunizing capacity of the immunogen was assessed from results on the recognition of the homologous conjugate – a coating or tracer conjugate carrying the same hapten as the immunizing conjugate – and those concerning the inhibition of the signal by quinoxyfen. The immunoreagent concentrations that were required to achieve an $A_{\max}$ value between 0.8 and 1.5 in the absence of analyte were not much different for the four antibodies (Table 1). However, a slightly higher affinity to quinoxyfen was observed with QXt-type antibodies. The lowest IC_{50} value was achieved, in both cELISA formats, with an antibody raised from hapten QXt. Particularly, antibody QXt-2 showed an IC_{50} value of 8.7 nM with the homologous coating conjugate in the indirect cELISA. This result suggests that haptens with a triple bond resulting from a Sonogashira cross-coupling reaction are convenient derivatives for high-affinity and specific antibody production.

Specificity of the generated antibodies was assessed by indirect cELISA using the homologous coating conjugate. Cross-reactivity was evaluated with a large series of compounds potentially present in real samples, such as fenhexamid, fluopyram, penthiopyrad, azoxystrobin, pyraclostrobin, boscalid, pyrimethanil, cyprodinil, fluopicolide, imidacloprid, and fludioxonil. No inhibition was observed at 10^4 nM analyte concentration, indicating that the antibodies were highly specific of quinoxyfen.

3.4. Immunoassay selection

All of the antibodies were also evaluated in combination with the corresponding heterologous conjugate – a coating or tracer conjugate carrying a hapten with the only difference of the triple bond in the linker, as compared to the immunizing conjugate. Optimum assay conditions were those affording an $A_{\max}$ between 0.8 and 1.5. It was found that all four antibodies bound the heterologous conjugate (Table S1). Moreover, assay sensitivity was enhanced in every case. The best IC_{50} value (around 5 nM) was achieved with antibody QXt-2

combined with the heterologous conjugate OVA–QXf in the conjugate-coated indirect cELISA format. This immunoassay showed a low limit of detection (0.18 nM), so it was selected for quinoxifen analysis in environmental samples.

3.5. Water sample analysis

Environmental water samples were collected from different origins in an intensive agricultural area. Sample I was obtained from the Albufera Lake in Valencia (Spain) and samples II and III were from two sewage treatment plants at Valencia City. These samples are characterized by potential contamination with agrochemicals. The pH of the samples was between 7.3 and 7.8, and the conductivity was around 1300 $\mu\text{S}/\text{cm}$. Samples were fortified with quinoxifen from a concentrated stock solution in *N,N'*-dimethylformamide and diluted in MilliQ water before they were analyzed by the developed indirect cELISA. A quinoxifen standard curve prepared in water was run in each microplate. Standards and blank samples were analyzed in triplicate wells and the mean absorbance values were interpolated into the standard curve run in the same plate. The obtained recovery values and coefficients of variation are listed in Table 2. From this study, it was observed that the selected immunoassay could accurately and precisely measure quinoxifen at 60 $\mu\text{g}/\text{L}$ in environmental water samples after a simple dilution in water. This limit of quantification is higher than the values commonly obtained by instrumental methods; however, this immunochemical method could be a valuable analytical complement for rapid and economic monitoring of quinoxifen as well as for *in-situ* analysis. The described immunoreagents are promising candidates for the development of a variety of immunochemical analytical tools, such as competitive ELISA, affinity columns, dipsticks, biosensors, etc.

4. Conclusions

High-affinity and specific antibodies can be generated from a hapten that holds an alkynyl hydrocarbon chain as spacer arm. Bioconjugates were prepared with high yields using the

purified active ester of the haptens. As far as we know, these are the first reported haptens, bioconjugates, and antibodies to this relevant pollutant. The **potential** applicability of these immunoreagents for sensitive and rapid environmental sample analysis has been shown.

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Abbreviations

$A_{\max}$: maximum absorbance; BSA: bovine serum albumin; cELISA: competitive enzyme-linked immunosorbent assay; HRP: horseradish peroxidase; IC_{50} : analyte concentration that inhibits 50% the immunochemical reaction; OVA: ovalbumin.

Appendix A. Supplementary data

Supplementary data related to this article can be found at

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Highlights

- Quinoxifen is a priority hazard pollutant in the European Water Framework Directive.
- Functionalized derivatives were synthesized for covalent conjugation to proteins.
- Bioconjugates and high-affinity antibodies specific of quinoxifen were prepared.
- A sensitive immunoassay (LOQ = 60 µg/L) was optimized and developed.
- Precision and accuracy of the method were verified with environmental water samples.

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Based on the above considerations, we have designed two haptens of quinoxifen, namely QXf and QXt (Fig. 1). The former incorporates a linear saturated hydrocarbon chain with a terminal carboxylate group for conjugation to the proteins, while the second has a carboxylated spacer arm of the same length with a triple bond that joins it to the C-4 position of the phenyl ring. The presence of the linear, rigid, triple carbon-carbon bond in the hydrocarbon chain results in a less flexible spacer arm. In a previous study, we evaluated, although with a much simpler molecule (Esteve-Turrillas et al., 2015), the influence of this type of spacer arm over the immune response; however, the results were not clearly conclusive although they suggested that a spacer arm with a triple carbon–carbon bond could favor the production of antibodies with superior affinity.

The syntheses of the above mentioned haptens was undertaken from an iodinate analogue of quinoxifen – aryl iodide **3** – readily available from 4,5,7-trichlorquinoline (**1**), and are based on the use of a palladium catalyzed Sonogashira cross-coupling reaction for the incorporation of the carboxylated hydrocarbon chain at the C-4 position of the phenyl ring (Supplementary

[Material](#)). Pure haptens QXt and QXf were obtained from **1**, as trifluoroacetate quinolinium salts, through a sequence of three and four steps and with an overall yield of 58% and 55%, respectively ([Fig. 1](#)). As in previous studies, the Sonogashira cross-coupling reaction was demonstrated to be an effective strategy to prepare functionalized haptens with the spacer arm at a halogenated aromatic ring (Parra et al., 2011; López-Moreno et al., 2014; Esteve-Turrillas et al., 2015).

3.2. Bioconjugate preparation

Carboxylated haptens were activated by the formation of the corresponding *N*-hydroxysuccinimidyl ester using *N,N*-disuccinimidyl carbonate and Et₃N in anhydrous acetonitrile ([Fig. 1](#)). The activation reaction proceeded from the trifluoroacetate quinolinium salts of the haptens, leading to the highly pure *N*-hydroxysuccinimidyl esters QXf-NHS (35.7 mg from 40.8 mg of hapten QXf, 90.4% yield) and QXt-NHS (27.9 mg from 25.9 mg of hapten QXt, 91.7% yield).

Since pure activated haptens were employed, the conjugates were readily accomplished with high yields. Moreover, a single coupling procedure was followed for both immunizing and assay conjugates in comparison to the classic one-pot coupling strategy in which two different activation methods are recommendable. The obtained hapten-to-protein molar ratios for hapten QXt were 17.3, 4.1, and 1.7 for BSA, OVA, and HRP conjugates, respectively. Those of hapten QXf were 14.5, 3.1, and 1.3 for BSA, OVA, and HRP conjugates, respectively. These values were within the expected optimum range for immunizing and assay conjugates. MALDI spectra of the prepared conjugates can be found as [Supplementary Material](#) ([Fig. S2](#), [S3](#) and [S4](#)).

3.3. Antibody characterization

Two antibodies were produced from each of the two BSA conjugates carrying the synthetic quinoxifen haptens. Antibodies QXt-1 and QXt-2 were generated with the conjugate of hapten

QXt, and antibodies QXf-1 and QXf-2 were raised with the conjugate BSA-QXf. The immunizing capacity of the immunogen was assessed from results on the recognition of the homologous conjugate – a coating or tracer conjugate carrying the same hapten as the immunizing conjugate – and those concerning the inhibition of the signal by quinoxifen. The immunoreagent concentrations that were required to achieve an $A_{\max}$ value between 0.8 and 1.5 in the absence of analyte were not much different for the four antibodies (Table 1). However, a slightly higher affinity to quinoxifen was observed with QXt-type antibodies. The lowest IC_{50} value was achieved, in both cELISA formats, with an antibody raised from hapten QXt. Particularly, antibody QXt-2 showed an IC_{50} value of 8.7 nM with the homologous coating conjugate in the indirect cELISA. This result suggests that haptens with a triple bond resulting from a Sonogashira cross-coupling reaction are convenient derivatives for high-affinity and specific antibody production.

Specificity of the generated antibodies was assessed by indirect cELISA using the homologous coating conjugate. Cross-reactivity was evaluated with a large series of compounds potentially present in real samples, such as fenhexamid, fluopyram, penthiopyrad, azoxystrobin, pyraclostrobin, boscalid, pyrimethanil, cyprodinil, fluopicolide, imidacloprid, and fludioxonil. No inhibition was observed at 10^4 nM analyte concentration, indicating that the antibodies were highly specific of quinoxifen.

3.4. Immunoassay selection

All of the antibodies were also evaluated in combination with the corresponding heterologous conjugate – a coating or tracer conjugate carrying a hapten with the only difference of the triple bond in the linker, as compared to the immunizing conjugate. Optimum assay conditions were those affording an $A_{\max}$ between 0.8 and 1.5. It was found that all four antibodies bound the heterologous conjugate (Table S1). Moreover, assay sensitivity was enhanced in every case. The best IC_{50} value (around 5 nM) was achieved with antibody QXt-2

combined with the heterologous conjugate OVA-QXf in the conjugate-coated indirect cELISA format. This immunoassay showed a low limit of detection (0.18 nM), so it was selected for quinoxifen analysis in environmental samples.

3.5. Water sample analysis

Environmental water samples were collected from different origins in an intensive agricultural area. Sample I was obtained from the Albufera Lake in Valencia (Spain) and samples II and III were from two sewage treatment plants at Valencia City. These samples are characterized by potential contamination with agrochemicals. The pH of the samples was between 7.3 and 7.8, and the conductivity was around 1300 $\mu\text{S}/\text{cm}$. Samples were fortified with quinoxifen from a concentrated stock solution in *N,N'*-dimethylformamide and diluted in MilliQ water before they were analyzed by the developed indirect cELISA. A quinoxifen standard curve prepared in water was run in each microplate. Standards and blank samples were analyzed in triplicate wells and the mean absorbance values were interpolated into the standard curve run in the same plate. The obtained recovery values and coefficients of variation are listed in [Table 2](#). From this study, it was observed that the selected immunoassay could accurately and precisely measure quinoxifen at 60 $\mu\text{g}/\text{L}$ in environmental water samples after a simple dilution in water. This limit of quantification is higher than the values commonly obtained by instrumental methods; however, this immunochemical method could be a valuable analytical complement for rapid and economic monitoring of quinoxifen as well as for *in-situ* analysis. The described immunoreagents are promising candidates for the development of a variety of immunochemical analytical tools, such as competitive ELISA, affinity columns, dipsticks, biosensors, etc.

4. Conclusions

High-affinity and specific antibodies can be generated from a hapten that holds an alkynyl hydrocarbon chain as spacer arm. Bioconjugates were prepared with high yields using the

purified active ester of the haptens. As far as we know, these are the first reported haptens, bioconjugates, and antibodies to this relevant pollutant. The potential applicability of these immunoreagents for sensitive and rapid environmental sample analysis has been shown.

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Abbreviations

$A_{\max}$: maximum absorbance; BSA: bovine serum albumin; cELISA: competitive enzyme-linked immunosorbent assay; HRP: horseradish peroxidase; IC_{50} : analyte concentration that inhibits 50% the immunochemical reaction; OVA: ovalbumin.

Appendix A. Supplementary data

Supplementary data related to this article can be found at

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Table 1
Antibody characterization by two cELISA formats using the homologous conjugate (n = 3).

Assay format	pAb	Antibody titer	[C] ^a	Parameter		
				A _{max}	Slope	IC ₅₀ (nM)
Direct	QXt-1	30000	10	0.81 ± 0.03	0.76 ± 0.09	34 ± 6
	QXt-2	10000	10	0.96 ± 0.14	0.56 ± 0.10	59 ± 11
	QXf-1	10000	30	1.50 ± 0.15	0.46 ± 0.06	623 ± 116
	QXf-2	10000	30	1.50 ± 0.09	0.46 ± 0.03	256 ± 89
Indirect	QXt-1	3000	100	1.32 ± 0.19	1.04 ± 0.09	30 ± 1
	QXt-2	3000	100	1.03 ± 0.18	0.70 ± 0.04	8.7 ± 0.1
	QXf-1	3000	100	1.21 ± 0.27	0.75 ± 0.02	76 ± 17
	QXf-2	3000	100	1.01 ± 0.22	0.74 ± 0.12	22 ± 9

^a Conjugate concentrations are in µg/L.

Table 2
Recovery values from spiked water samples (n = 3).^a

	Spiked (µg/L)	Recovery (%)	CV (%)
Sample I	60	90.0	6.5
	200	92.5	6.6
	600	95.5	12.9
Sample II	60	97.4	4.1
	200	101.9	20.2
	600	95.5	18.5
Sample III	60	110.6	3.6
	200	89.9	5.7
	600	91.5	6.9

^a Samples were diluted 100-fold with MilliQ water before they were assayed.

Figure
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FIGURE CAPTIONS

Fig. 1. Synthesis of quinoxifen haptens and the corresponding *N*-hydroxysuccinimidyl esters.

Inserts: Molecular structure of quinoxifen and global minimum-energy conformer of quinoxifen. Calculations were performed using Molecular Mechanics (MM3) as implemented in the CAChe program [CAChe WorkSystem Pro software, version 7.5.0.85 (Fujitsu Ltd, Tokyo, Japan)]. A systematic conformational search was performed (all rotatable bonds were rotated by 15 degree steps).

Supplementary Material

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Quinoxifen