Chemical Research in Toxicology

ARTICLE

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Synthetic Approach to Gain Insight into Antigenic Determinants of Cephalosporins: In Vitro Studies of Chemical Structure−IgE Molecular Recognition Relationships

Maria Isabel Montañez,[†,‡] Cristobalina Mayorga,[‡] Maria Jose Torres,[§] Adriana Ariza,[‡] Miguel Blanca,[‡,§] and Ezequiel Perez-Inestrosa[*,†]

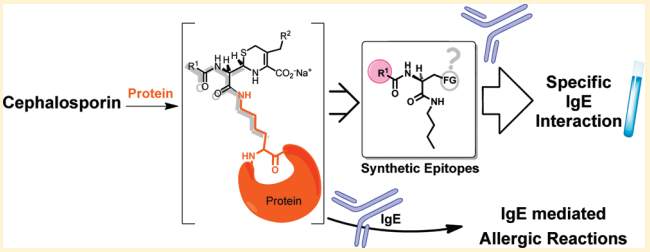
[†]Department of Organic Chemistry, Faculty of Science, University of Malaga, 29071 Malaga, Spain

[‡]Research Laboratory, IMABIS Foundation-Carlos Haya Hospital, 29009 Malaga, Spain

[§]Allergy Service, Carlos Haya Hospital, 29009 Malaga, Spain

Supporting Information

ABSTRACT: Cephalosporins, after penicillins, are the most widely used antibacterial agents in infectious diseases and the cause of adverse immune reactions in the world. Whether a patient with a suspected allergy to a β-lactam can safely take a cephalosporin is often a matter of debate. However, there are no tests with enough sensitivity to detect allergy to cephalosporins. Understanding the way in which the drug metabolizes after protein conjugation is important if we are to make advances in the diagnosis of clinical allergy. Structural studies of cephalosporin−protein adducts have never been addressed successfully and are difficult to investigate. Our approach to determine the requirements involved in antigenic determinant structures consisted of designing and synthesizing a proposed skeleton that remains linked to the carrier protein after chemical degradation in cephalosporin conjugated to carrier proteins. In this study, a series of proposed epitopes were efficiently synthesized following a versatile methodology, involving the condensation of the R^1 acyl side chains of native cephalosporins, with the nuclear fragment structures (derived from amino acids or other aminofunctionalized molecules). The final well-defined structures **1**−**4** (**a**−**f**), representing a fragment from the proposed cephalosporin−Lys(protein) adduct intermediate, consist of closely related low-molecular-weight molecules, differing only in the functional group at C-3 and the R^1 side chains. They were assessed with sera from patients allergic to cephalosporins to study structure−IgE molecular recognition relationships. These IgE showed an enhanced recognition to proposed new skeleton epitopes with adequate functionality at C-3, with the specifities mainly related to the R^1 acyl side chain. Thus, this study led us to refine the model haptenic structures of cephalosporins and gain insight into the chemical mechanism of epitope formation.



INTRODUCTION

Cephalosporins are widely prescribed antibiotics and represent, after penicillins, the most common cause of adverse drug reactions [1,2] mediated by specific immunological mechanisms. IgE-mediated hypersensitivity reactions to penicillins have been extensively studied and used as a model to increase understanding of the immunological mechanisms involved in these reactions. Penicillins are chemically reactive small allergenic molecules, and according to the hypothesis of Landsteiner,[3] only when they covalently bind to protein, through reaction of the β-lactam ring with the nucleophilic free amino groups on proteins,[4] can the so formed hapten−protein conjugate induce an immune response. The mechanism of cephalosporin-induced allergic reactions fits the hapten hypothesis also due to its β-lactam reactivity. However, unlike well-established structures responsible for penicillin allergy, the metabolites involved in the immunological responses to cephalosporins are still unknown. This has limited adequate evaluation of allergic reactions to these antibiotics and the study of molecular recognition by specific IgE antibodies.[5] As a consequence, attempts to develop in vivo and in vitro tests to evaluate allergy to cephalosporins have been unsuccessful, and skin testing including just the native drug has to be used as the skin test agent to detect antibodies specific to these drugs.[6,7]

Penicillins and cephalosporins share structural similarities; they possess a β-lactam ring which has the antimicrobial activity and diverse side chains (Scheme 1). However, they differ in their immunologic behavior, determined by their intrinsic chemical reactivity, which is related with the electrophilic properties of the β-lactam carbonyl,[8] and therefore the possibility to bind to proteins with subsequent epitope formation. The main difference between cephalosporins and penicillins is the ring to which the β-lactam is fused. A five-membered thiazolidine ring in penicillins

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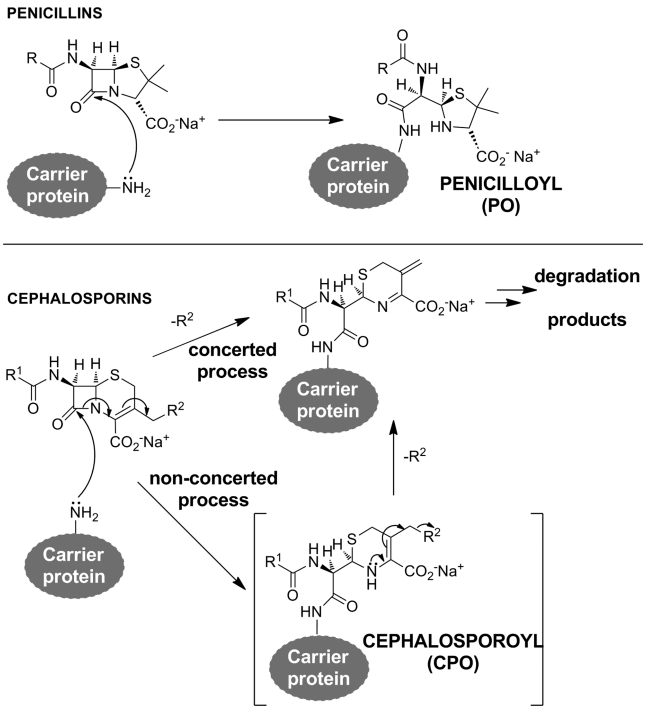
Scheme 1. Nucleophilic Ring-Opening of β-Lactams by Proteins and Subsequent Formation of Antigenic Determinants[a]

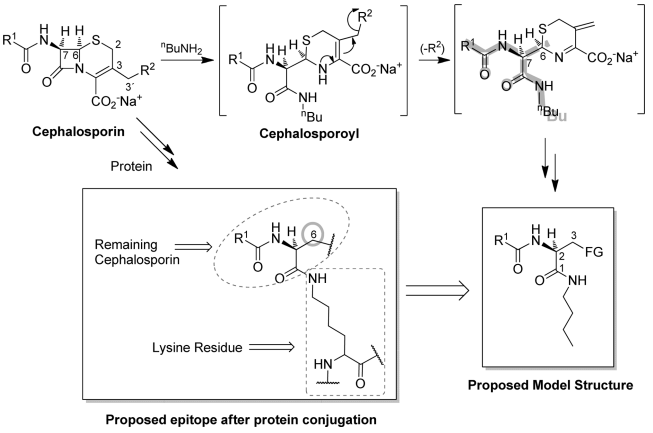


[a] Unlike well-established structures responsible for allergy to penicillin and penicilloyl, the equivalent chemical structures of cephalosporin antigenic determinants are still unknown.

and a six-membered dihydrothiazine ring in cephalosporins provide different degradation patterns. In penicillins, the higher tension within the β-lactam ring compared to that in cephalosporins results in a more increased chemical reactivity.[9] Haptenization of proteins by penicillins takes place quickly and efficiently, obtaining a conjugate, penicilloyl (PO; Scheme 1), which is stable enough to be isolated and characterized by spectroscopic techniques. In cephalosporins, the lower reactivity of the β-lactam ring slows the haptenization process. The R^2 chemical structure modulates this reactivity depending on its ability to polarize electronic binding. Most clinically relevant cephalosporins have a good leaving group at the 3′ position which increases the reactivity of the β-lactam via the elimination of R^2. The departure of R^2 has been interpreted in terms both of concerted[10−14] and nonconcerted[15,16] with the β-lactam ring-opening (Scheme 1). Whatever the process, the opening of the β-lactam leads to a well-evidenced departure of the good R^2 leaving group. The resulting conjugate is unstable and suffers degradation through dihydrothiazine ring rupture.

Despite the lack of knowledge regarding the chemical structures derived from cephalosporins which interact specifically with the variable region of antibodies, it is accepted that the cephalosporin is conjugated to the carrier molecule. Evidently, uncertainty exists about the epitope structure, which could involve either the complete cephalosporin structure or just parts of it after degradation. This unresolved question, as well as whether R^1 and/or R^2 are part of the antigenic determinant, will have important implications in the IgE response and, moreover, in further clinical evaluation with both in vivo and in vitro assays.

Scheme 2. Proposed Aminolysis Pathway and Resulting Degradation Products for Cephalosporins



Whatever the case, there is clinical evidence that the R^1 side chain may contribute to IgE induction and cross-reactive responses. In fact, some patients can react selectively to the cephalosporin involved in the reaction, whereas others react to different cephalosporins which have the same or a similar R^1 side chain,[17−19] and a third group of patients has cross-reactivity with other β-lactams, especially penicillins, which have the same or a similar R^1.[6,18,20−22] The study of allergy to cephalosporins has classically focused on the assessment of tolerance in patients primarily sensitized to penicillin derivatives.[6,20−24] However, few studies have analyzed the problem of cross-reactivity in subjects primarily sensitized to cephalosporins and potentially allergic to penicillins.[17,18] Whether a patient with an allergic reaction to penicillins can safely take other β-lactams such as cephalosporin is a matter of debate and will depend on the β-lactam involved in the reaction.[17,18,25,26] However, cross-reactivity due to similarities in R^2 has only vaguely been described.[27]

Chemically, all attempts to study the reaction between cephalosporins with simple nitrogen nucleophiles have failed to identify an exact chemical structure. Results with butylamine as nucleophile suggest the cephalosporoyl conjugate formation and further fragmentation of the dihydrothiazine ring, leading to structures including at least the remaining β-lactam and the R^1 side chain (Scheme 2).[28]

Taking into account both the evidence of the departure of the R^2 group as a rule and the important role of the R^1 structure in specific immunologic recognition, we proposed that after opening the β-lactam ring by amino groups of proteins, the resulting structure is unstable; thus, the dihydrothiazine ring undergoes degradation and becomes lost. As a result, only R^1 and the amino acid residue included in the β-lactam moiety of the cephalosporin remain linked to the protein, and this hapten−carrier conjugate is the final structure that interacts with the immunological system (Scheme 2). Our approach to determine the structure of the epitope consisted of synthesizing the proposed skeleton that remains linked to the carrier protein after chemical degradation in the cephalosporin conjugate. To carry out this synthesis, butylamine was chosen as the nucleophilic amine model to emulate the primary amino groups of the side chains of lysine present in proteins. Thus, the proposed model structures, in agreement with our hypothesis, would result from the nucleophilic reaction of butylamine to cephalosporins. A number of

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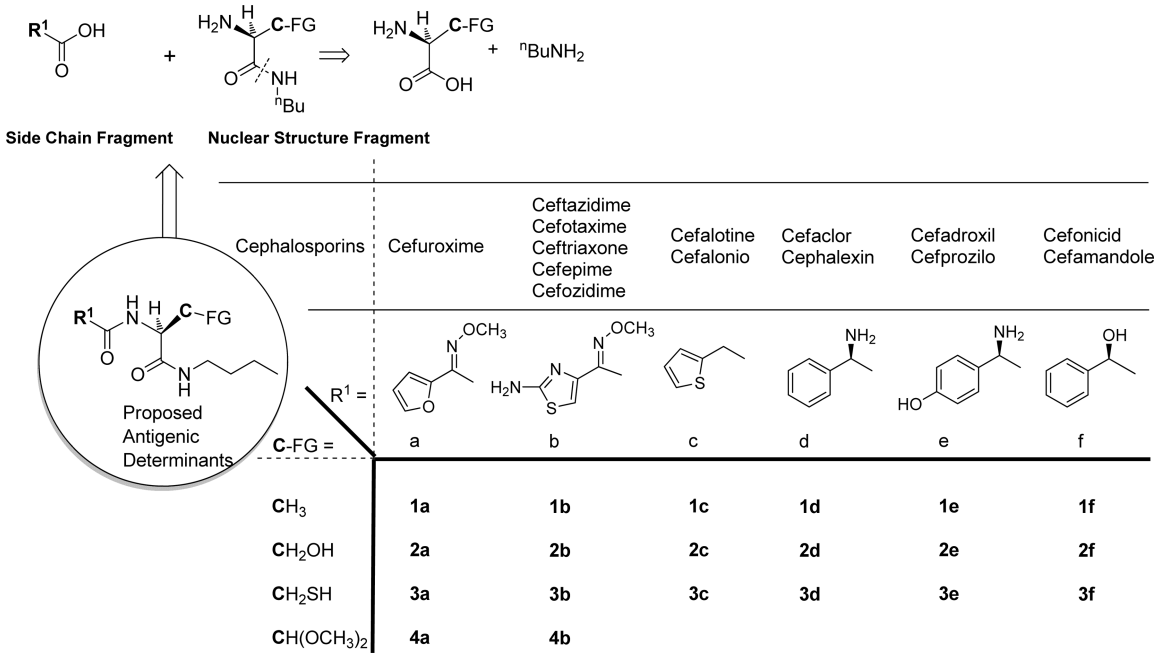


Figure 1. General retrosynthetic analysis for proposed antigenic determinants and description of all synthesized compounds, combining different R^1 side chains (**a**−**f**) and functional groups (FG) at C-3 (**1**−**4**).

chemically well-defined compounds (**1a**−**f**, Figure 1), proposed as models, were synthesized, and their recognition by IgE from cephalosporin allergic patients was evaluated by a radioallergosorbent test (RAST).[28] The immunological results indicated that the IgE antibodies recognize the proposed metabolites and that specificities were related mainly to the R^1 acyl side chain containing the culprit cephalosporin. The functional group in the 3-position was a methyl group, the most simple version of the proposed model structures, in order to establish the basis of the structural requirements for a specific IgE interaction. However, the C-3 atom in the model structure, derived from C-6 in the native cephalosporin, is originally like a carbonyl group and, as such, can suffer different reactions in the physiological media. We expected C-3 in our model antigenic determinants to present a higher oxidation state than that corresponding to a methyl group, by C-3 covalent binding to either oxygen or sulfur atom.

To confirm this assumption, we have extended our preliminary work on structural requirements for a cephalosporin epitope by assessing the recognition of new more complex molecules. The aim of the present study was to investigate how the functional group at C-3 in the proposed antigenic determinant model structures can modulate their recognition by specific IgE antibodies. The synthesis of a series of proposed antigenic determinants (Figure 1), all containing the same basic structure but with variations in both the substituent R^1 and different functional groups at the C-3 position, including hydroxyl, thiol, and acetal derived, is described. Obtaining pure and chemically well-defined products allows one to study the specificity of IgE antibodies from patients with an immediate allergic reaction to cephalosporin. In vitro clinical assays were performed to establish the recognition of different synthesized structures by IgE antibodies specific to cephalosporins.

EXPERIMENTAL PROCEDURES

Chemicals. All materials were obtained from commercial suppliers and used without further purification. Solvents were distilled when anhydride conditions were needed.[29] Column chromatography was carried out on silica gel 60 (63−200 μm, Merck 7734). Organic solutions were dried with $MgSO_4$ and concentrated in vacuum.

Instrumentation and Mode of Analysis. TLC analyses were performed on silica gel 60 F254 (Merck 5719) or aluminum oxide 60 F254 (Merck 5550). As general criteria of purity, TLC analysis of all immunologically evaluated compounds was performed in two different sets of conditions, and a single spot was always observed. Melting points were determined on a Gallenkamp instrument and are given uncorrected. MS (EI) were recorded on an HP-MS 5988A spectrometer operating at 70 eV or Thermo Finnigan Trace at 70 eV. HRMS were recorded on an AutoSpecE, CACTI, University of Vigo (Spain). NMR spectra were recorded on a Bruker ARX-400 at 400 MHz for ^1H and 100.6 MHz for ^{13}C. Chemical shifts are given relative to the residual signal of solvents, δ H 7.24 ppm and δ C 77.0 ppm for deuteriochloroform or δ H 3.31 ppm and δ C 49.0 ppm for deuteriomethanol. Optical rotations were determined by using a Perkin-Elmer 241 digital polarimeter. Elemental analyses were carried out in the Microanalytical Laboratory, SCAI, University of Malaga.

General Procedures for Amide Formation (Coupling Reactions). *Generel Procedure A.* To a stirred solution of *N*-(*tert*-butoxycarbonyl)-L-amino acid (1 equiv.) in anhydrous $CHCl_3$ at 0 °C, *N*-methylmorpholine (1.1 equiv.) and isobutylchloroformate (1.1 equiv.) were sequentially added dropwise. After 1 h, butylamine (1.2 equiv.) was added, and the reaction was slowly warmed to room temperature. After 40 h of reaction, the mixture was washed with H_2O, 5% HCl aqueous solution, H_2O, 10% $NaHCO_3$ aqueous solution, and H_2O. After workup, pure compounds were obtained.

Generel Procedure B. To a solution of acid **16a**−**f** (1 equiv.) in anhydrous CH_2Cl_2 (2 mL per mmol) in an ice bath were added successively recrystallized *N*-hydroxybenzotriazole (HOBT) (1.2 equiv.) in DMF/CH_2Cl_2 1:1 (1 mL per mmol) and *N*,*N*′-dicyclohexylcarbodiimide (DCC) (1 equiv) in anhydrous CH_2Cl_2 (1 mL per mmol). The mixture was stirred for 2 h at room temperature under argon. Then, amine (1 equiv. of **7** + *N*-methylmorpholine (NMM), **10**, or **15**) solution in CH_2Cl_2 (2 mL per mmol) was added dropwise, and the mixture was stirred for 40 h at room temperature. Most of the dicyclohexylurea was

removed by filtration and washed with a small amount of CH_2Cl_2. The solvents were evaporated. The remaining syrup was dissolved in CH_2Cl_2, washed with 10% $NaHCO_3$ aqueous solution, dried ($MgSO_4$), and evaporated.

Generel Procedure C. The same procedure as that described in General Procedure B was followed using the following reagents and workup: acid **16a**−**f** (1 equiv.) in anhydrous CH_2Cl_2 (2 mL per mmol), HOBT (1.1 equiv.) in DMF/CH_2Cl_2 1:1 (1 mL per mmol), 1--(3-(dimethylamino)propyl)-3-ethyl-carbodiimide hydrochloride (EDCI) (1 equiv.) in anhydrous CH_2Cl_2 (5 mL per mmol), and amine (1 equiv. of **7** + NMM, **10**, or **15**) solution in CH_2Cl_2 (2 mL per mmol). After stirring for 24 h at room temperature, solvents were evaporated. The remaining syrup was dissolved in CH_2Cl_2, washed with 10% HCl aqueous solution, 10% $NaHCO_3$ aqueous solution and H_2O, dried ($MgSO_4$), and evaporated.

General Procedure for the Hydrolysis of the Trityl Group. The N-tritylated compound was treated with a 50% formic acid aqueous solution for 30 min at 70 °C. The mixture was then cooled to room temperature, and water was added. Triphenylcarbinol was separated by filtration and washed with water. The filtrate was concentrated to dryness under reduced pressure, and the residue was triturated with Et_2O. The solid was filtered and dried to obtain a pure product.

General Procedure for the Hydrolysis of the *tert*-Butoxycarbonyl Group. *General Procedure D.* To a stirred solution of the corresponding compound in EtOAc, at room temperature, a 3 M HCl aqueous solution was added. After 5 h, the solvent was evaporated in vacuum, and the oily residue was triturated with Et_2O affording the product as a white solid in quantitative yield.

General Procedure E. To a stirred solution of the corresponding compound in CH_2Cl_2, at room temperature, trifluoroacetic acid was added. After 4 h, the solvent was evaporated in vacuum, and the oily residue was triturated with Et_2O affording the product as a white solid in quantitative yield.

General Procedure for Disulfide Reduction. *General Procedure F.* A solution of the disulfide (1 equiv.), triethylamine (TEA) (2 equiv.), and DTT (4 equiv.) in degassed solvent was stirred for 1 h at room temperature under argon. CH_2Cl_2 was the solvent used for side chains **a** and **c**; MeOH was used for **b** and **e**. When the solvent was MeOH, it was evaporated, and the mixture was dissolved in EtOAc. The organic phase was washed with water and dried over $MgSO_4$.

General Procedure G. A solution of the disulfide (1 equiv.) and DTT (4 equiv.) in degassed water was stirred for 10 h at room temperature under argon. Then, several drops of concentrated HCl were added, and the solution was stirred for 10 min. The aqueous phase was washed with CH_2Cl_2 (×3) and evaporated in vacuum, and the resultant residue was triturated with degassed ethyl ether.

Amine Synthesis: Nuclear Structure Fragments

Compound 6 (N-Boc-Ser-NH^{n}Bu). This was obtained following general procedure A for amide formation starting from compound **5** (1.4 g, 6.9 mmol). It recrystallized from EtOAc−hexane as a white crystalline solid (1.4 g, 77%).

Compound 7 (Ser-NH^{n}Bu). To a stirred solution of compound **6** (1.4 g, 5.4 mmol) in EtOAc (6 mL), at room temperature, a 3 M HCl aqueous solution was added. After 3 h, the solvent was evaporated in vacuum, and the oily residue was triturated with Et_2O to obtain the hydrochloride product as a colorless oil (0.94 g, 88%).

Compound 9 (N-Boc-Cys-NH^{n}Bu)$_2$. This was obtained following general procedure A for amide formation starting from compound **8** (2.6 g, 6 mmol). Recrystallization from $CHCl_3$−EtOAc gave a white solid (1.6 g, 99%).

Compound 10 (Cys-NHnBu)$_2$. To a stirred solution of compound **9** (2.4 g, 4.35 mmol) in CH_2Cl_2 (25 mL), at room temperature, trifluoroacetic acid (2 equiv) was added. After 20 h, 75 mL of CH_2Cl_2 was added. The solution was neutralized with NaOH aqueous solution, washed with H_2O, and dried over $MgSO_4$. Solvent evaporation yielded a white solid (97%). Recrystallization was from EtOAc−Hexane.

Compound 12. This was synthesized as previously described.[30]

Compound 13. An excess of *n*-butylamine (5.75 mL, 58.1 mmol) was added dropwise to stirred bromo acetal **12** (6 g, 26.4 mmol) under argon at 5 °C. After addition, the reaction mixture was allowed to reach room temperature and stirred for 24 h. Then, the mixture was concentrated under reduced pressure. The residue was dissolved in $CHCl_3$, washed with 1 N HCl, dried over $MgSO_4$, and concentrated under reduced pressure to give a yellow solid (6.38 g, 90%) corresponding to the desired product.

Compound 14. A mixture of **13** (2 g, 7.5 mmol) and benzyl amine (3.25 mL, 29.8 mmol) was stirred and heated to 110 °C for 48 h. After the reaction, the mixture was allowed to reach room temperature, a minimum volume of cold $CHCl_3$ was added, and the mixture was filtered. The filtrate was dissolved with more $CHCl_3$ (50 mL), washed with H_2O (×3), dried ($MgSO_4$), and concentrated under reduced pressure. The crude product was purified by column chromatography on silica gel (Hexane/EtOAc, 1:1) affording **14** as a yellow oil (2.1 g, 95%).

Compound 15. A suspension of **14** (0.8 g, 2.7 mmol) and Pearlman catalyst (0.67 g) in MeOH (90 mL) was hydrogenized for 48 h at 90 psi at room temperature. The mixture was filtered through Celite, and the solvent was eliminated to afford the desired amine **15** as a yellow liquid (99%).

Synthesis/Protection of Side Chain Acid: Side Chain Fragment

Compound 16a. 2-Oxo-furanacetic acid (840 mg, 6 mmol) was treated with methoxylamine hydrochloride (1 g, 12 mmol) and Na_2CO_3 (2.5 g, 24 mmol) in anhydrous MeOH. The mixture was refluxed for 90 min. When the crude reaction reached room temperature, concentrated HCl was added until the mixture reached pH 3. The solvent was concentrated, and the resulting residue was extracted with Et_2O (70 mL and 3 × 25 mL). The organic phases were dried ($MgSO_4$) and concentrated under reduced pressure affording a 1:4 *E*/*Z* mixture of **16a** as a brown solid (0.91 g, 84%). Recrystallization from benzene gave the pure isomer *Z*-**16a** as a white crystalline solid (364 mg, 36% global yield).

Compound 16b. A solution of 2-(2-amino-1,3-thiazol-4-yl)-2-(methoxyimino) acetic acid (6 g, 29.8 mmol) and TEA (9.2 mL, 65.6 mmol) in $CHCl_3$/DMF 2:1 (150 mL) was cooled to 0 °C. Then trityl chloride (9.14 g, 32.8 mmol) was added in small portions for one hour. The mixture was slowly warmed to room temperature overnight. Afterward, 1 M HCl aqueous solution was added, and the mixture was extracted with $CHCl_3$. After drying ($MgSO_4$) and solvent evaporation, a white solid was obtained (10.7 g, 81%).

Compounds 16d and 16e. A solution of di-*tert*-butyldicarbonate (1.1 equiv.) in acetone was slowly added to a stirred solution of the corresponding compound (1 equiv.) and TEA (1 equiv.) in acetone/H_2O 1:1 at room temperature. The mixture was stirred for 24 h, and the acetone was evaporated. The aqueous phase was acidified to pH 2 with 10% HCl aqueous solution and extracted with Et_2O (×2). The combined ethereal extracts were dried over $MgSO_4$, filtered, and evaporated in vacuum yielding the corresponding pure compound.

*Compounds **2**−**4** (**a**−**f**): Model Structures*

Compound 2a. This was obtained following general procedure B described for amide formation starting from **16a** (338 mg, mmol). The crude product was purified by column chromatography on silica gel (hexane/EtOAc, 4:6) affording **2a** as a white solid (250 mg, 40%). It was recrystallized in benzene.

Compound 17b. This was obtained following general procedure B described for amide formation starting from **16b** (887 mg, 2 mmol). The crude product was purified by column chromatography on silica gel (EtOAc) affording **17b** as a yellow solid (730 mg, 63%). It was recrystallized in EtOAc−hexane.

Compound 2b. This was obtained following the general procedure described for hydrolysis of trityl group, starting from **17b** (0.68 g, 1.2 mmol). Yellow solid (0.3 g, 75%).

Compound 2c. This was obtained following the general procedure B described for amide formation starting from **16c** (284 mg, 2 mmol). The crude product was purified by column chromatography on silica gel (hexane/EtOAc, 1:1) affording **2c** as a white solid (250 mg, 45%). It was recrystallized in $CHCl_3$−hexane.

Compound 17d. This was obtained following general procedure B described for amide formation starting from **16d** (948 mg, 7 mmol). The crude product was purified by column chromatography on silica gel (hexane/EtOAc, 4:6) affording **17d** as a white solid (760 mg, 32%). It was recrystallized in EtOAc−hexane.

Compound 2d. This was obtained following procedure D described for the hydrolysis of the Boc group, starting from **16d**; white solid.

Compound 17e. This was obtained following general procedure B described for amide formation starting from **16e** (512 mg, 1.9 mmol). The crude product was purified by column chromatography on silica gel (hexane/EtOAc, 1:1) affording **17e** as a white solid (370 mg, 47%).

Compound 2e. This was obtained following procedure D described for hydrolysis of the Boc group, starting from **17e**; white solid.

Compound 2f. This was obtained following general procedure B described for amide formation starting from **16f** (220 mg, 1.4 mmol). The crude product was purified by column chromatography on silica gel (hexane/EtOAc, 1:1 and then increasing the polarity EtOAc/MeOH, 9.5:0.5) affording **2f** as a white solid (100 mg, 23%).

Compound 18a. This was obtained following general procedure C described for amide formation starting from **16a** (507 mg, 3 mmol). The crude product was purified by flash chromatography ($CHCl_3$/MeOH, 10:0.15) affording **18a** as a white solid (570 mg, 58%). It was recrystallized in $CHCl_3$−benzene−hexane.

Compound 3a. This was obtained following disulfide reduction procedure F described, starting from **18a** (0.3 g, 0.46 mmol). After flash chromatography ($CHCl_3$/MeOH, 10:1.5), the pure compound was obtained. It was recrystallized in benzene to obtain the compound as white needles (180 mg, 71%).

Compound 18b. This was obtained following general procedure C described for amide formation starting from **16b** (1.33 g, 3 mmol). The crude extract was purified by flash chromatography (hexane/EtOAc, 7:3) to give **18b** as a yellow solid (1.04 g, 58%). It was recrystallized in $CHCl_3$−hexane.

Compound 19b. Compound **18b** (0.37 g, 0.3 mmol) was treated with a 1:1 mixture of acetone/50% aqueous formic acid solution for 90 min at 70 °C. After reaching room temperature, acetone was evaporated, and then a yellow solid (0.17 g, 78%) was obtained, working-up the general procedure described for the hydrolysis of the trityl group.

Compound 3b. This was obtained following disulfide reduction procedure F as described, starting from **19b** (165 mg, 0.23 mmol). The product was purified by column chromatography (hexane/EtOAc, 1:1); white solid (130 mg, 79%).

Compound 18c. This was obtained following general procedure C described for amide formation starting from **16c** (428 mg, 3 mmol). Compound **18c** was obtained as a white solid (661 mg, 73%) from the crude product recrystallization with $CHCl_3$−hexane.

Compound 3c. This was obtained following disulfide reduction procedure F as described, starting from **18c** (0.42 g, 0.7 mmol). The product was purified by column chromatography ($CHCl_3$/MeOH, 10:0.5); white solid (0.3 g, 71%).

Compound 18d. This was obtained following general procedure C described for amide formation starting from **16d** (753 mg, 3 mmol). A white solid (946 mg, 77%) corresponding to compound **18d** precipitated with $CHCl_3$−Et_2O.

Compound 19d. This was obtained following general procedure B described for the hydrolysis of the *tert*-butoxycarbonyl group, starting from **18d**; white solid.

Compound 3d. This was obtained following disulfide reduction procedure G as described, starting from **19d** (280 mg, 0.45 mmol); white solid (0.19 g, 67%).

Compound 18e. This was obtained following general procedure C described for amide formation starting from **16e** (801 mg, 3 mmol). After flash chromatography (hexane/EtOAc, 1:1), the pure compound was obtained as a white solid (535 mg, 42%).

Compound 19e. This was obtained following general procedure E described for hydrolysis of *tert*-butoxycarbonyl group, starting from **18e**; white solid.

Compound 3e. This was obtained following disulfide reduction procedure F as described, starting from **19e** (0.16 g, 0.25 mmol). After flash chromatography ($CHCl_3$/MeOH, 10:0.3), the pure compound was obtained. It was recrystallized in benzene to give white needles (78 mg, 50%).

Compound 18f. This was obtained following general procedure C described for amide formation starting from **16f** (229 mg, 1.5 mmol). A white solid (150 mg, 32%) corresponding to compound **18f** was obtained after flash chromatography (hexane/EtOAc, 1:1) or recrystallization in MeOH−$CHCl_3$−hexane.

Compound 3f. This was obtained following disulfide reduction procedure F as described, starting from **18f** (0.16 g, 0.25 mmol). The pure compound was obtained after flash chromatography ($CHCl_3$: MeOH, 10:0.3); white solid (78 mg, 50%).

Compound 4a. This was obtained following general procedure B described for amide formation, starting from a mixture of E/Z isomers (relative proportion 1:4) of **16a** (507 mg, 3 mmol). The crude product was purified by column chromatography on silica gel ($CHCl_3$) affording an E/Z 1:4 mixture of **18a** (910 mg, 85%). The isomers were separated by column chromatography on silica gel ($CHCl_3$/MeOH, 10:0.5) to give the Z isomer as a white solid (665 mg, 62%).

Compound 20b. This was obtained following general procedure B described for amide formation, starting from **16b** (655 mg, 1.5 mmol). After flash chromatography ($CHCl_3$), the pure compound **20b** was obtained as a yellow solid (559 mg, 60%).

Compound 4b. This was obtained following the general procedure described for hydrolysis of the trityl group, starting from **20b** (0.75 g, 1.2 mmol); yellow solid (0.3 g, 60%).

Immunochemical Studies. *Subjects.* From the patients with a confirmed diagnosis of an immediate reaction to cephalosporins, we selected seven cases that showed high levels (greater than 7%) of specific IgE antibodies to the cephalosporin involved in the reaction measured by direct RAST. Data from these patients included in the study are shown in Table 1. These patients were diagnosed following the procedure described in the European Network of Drug Allergy (ENDA) protocol based on skin testing, in vitro tests, and a drug provocation test if necessary.[31] Three types of clinical manifestations had been experienced: anaphylactic shock, anaphylaxis, or urticaria. Anaphylaxis was considered to be the presence of several of the following symptoms: pruritus on palms or soles, later becoming generalized, generalized erythema, urticaria, dyspnea, difficulty speaking or swallowing, and/or tachycardia. If hypotension and/or loss of consciousness also appeared, an anaphylactic shock was considered. Urticaria was defined as rapidly evolving and transient pruriginous wheals occurring at different body sites.

The institutional review boards approved the study, and informed consent for the diagnostic procedures was obtained from all patients.

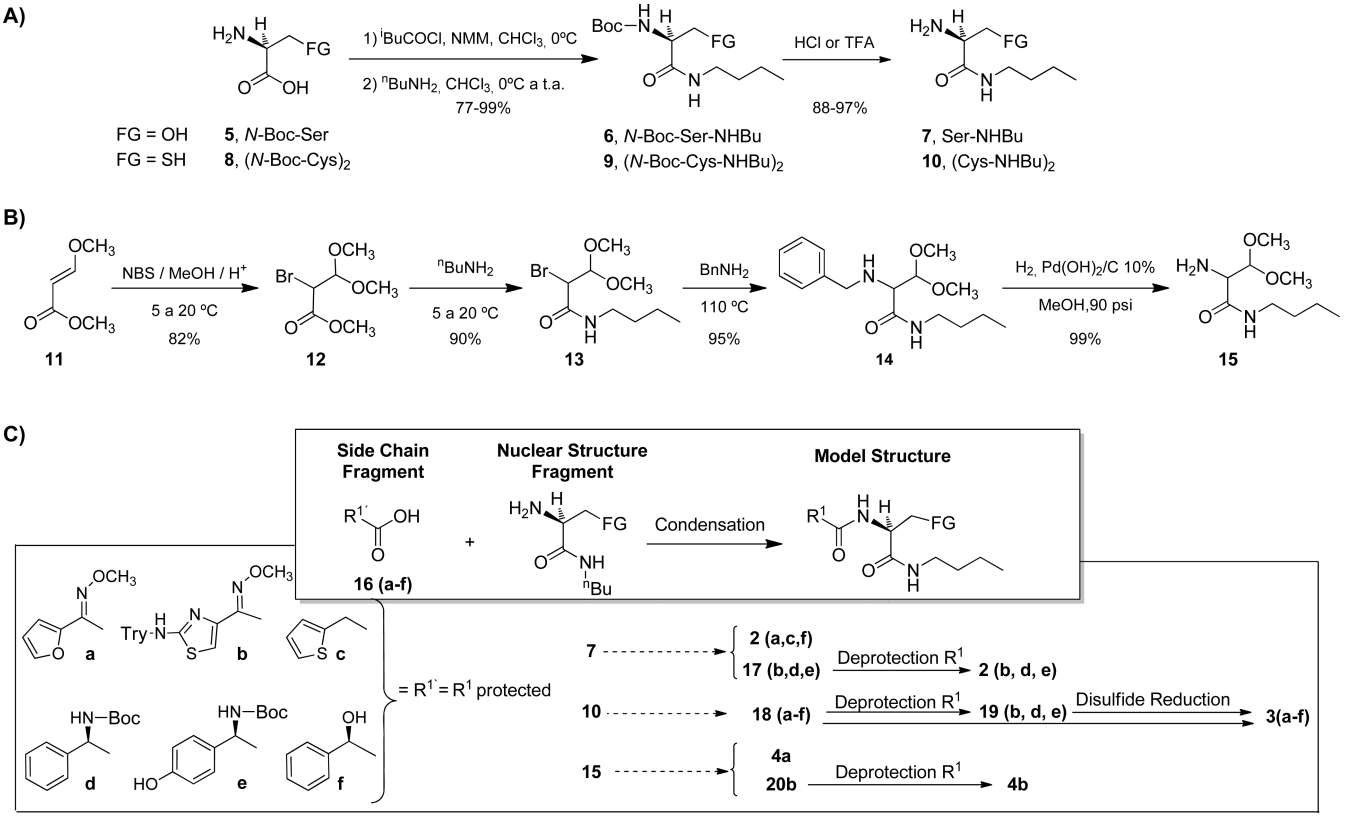
Skin Tests. Skin testing was carried out,[31,32] using 0.02 mL of solution prepared daily. The reagents and maximum concentrations accepted nowadays were benzylpenicilloyl-poly-L-lysine (PPL) (Diater Laboratories, Madrid, Spain) (5×10^{-5} M), minor determinant mixture (MDM) (Diater Laboratories, Madrid, Spain) (2×10^{-2} M), benzylpenicillin (Normon SA, Madrid, Spain) (105 IU/mL), amoxicillin (SB Smithkline Beecham, Madrid, Spain) (20 mg/mL), and ampicillin (Normon SA) (20 mg/mL). The following cephalosporins (2 mg/mL) were also used: cefaclor (Lilly, Madrid, Spain), cefuroxime (GlaxoSmithKline S.A, Madrid), ceftazidime (GlaxoSmithKline S.A),

Table 1. Clinical Characteristics of Allergic Patients and Skin Test Results with Different Cephalosporins

						skin tests								
case	age	sex	reaction	drug	INT[a]	cefuroxime	cefotaxime	cefozidime	ceftriaxone	cefepime	cefonicid	cefaclor	ceftazidime	cefadroxil
1	57	F	anaph.	ceftazidime	120	−	−	−	−	−	−	−	+	−
2	38	F	urticaria	cefotaxime	10	+	+	+	+	−	−	−	−	−
3	42	M	anaph.	cefuroxime	1	+	−	−	−	−	−	−	−	−
4	36	F	anaph.	cefuroxime	6	+	+	+	−	−	−	−	−	−
5	22	F	anaph.	cefuroxime	0.5	+	−	−	−	−	−	−	−	−
6	42	F	anaph.	cefuroxime	0.5	+	+	+	−	−	−	−	−	−
7	32	F	anaph.	cefuroxime	1	+	−	−	−	−	−	−	−	−

[a] INT, interval between the reaction and study (in months).

Scheme 3. Synthesis of (A,B) Nuclear Structure Fragments and (C) Model Haptenic Structures



ceftriaxone (Roche, Basel, Switzerland), cefotaxime (Roussel Iberica Laboratorios, Madrid, Spain), and cefonicid (GlaxoSmithKline S.A). Higher concentrations may cause nonspecific irritative reactions, even in subjects with good tolerance or no exposure to β-lactams.[31] In the skin prick tests, a wheal >2 mm was considered positive. In the intradermal tests, the wheal area was marked initially and 20 min after testing, and an increase in diameter greater than 3 mm was considered positive.[31,33]

Radioimmunoassay for IgE Determination. These were done by RAST to benzylpenicillin, amoxicillin, ampicillin, and to different cephalosporins (cefuroxime, cefotaxime, cefoxidime, ceftriaxone, cefepime, cefonicid, cefaclor, ceftazidime, and cefadroxil) conjugated to poly-L-Lysine (PLL) (Sigma, St. Louis, MO), as previously described.[32] Blood samples were obtained when patients were evaluated, and sera were kept at −20 °C until assayed. Briefly, 30 μL of the patient's sera was incubated in the solid phase with different β-lactam−PLL conjugates. After washing, radiolabeled anti-IgE antibody (Kindly provided by ALK-Abello Laboratories, Madrid, Spain) was added and incubated overnight. The discs were then washed, and their radioactivity was measured in a gamma counter, Cobra II autogamma (Packard BioScience Company, Frankfurt, Germany). Results were calculated as a percentage of the maximum and considered positive if they were higher than 2.5%, which was the mean +2SD of a negative control group.[32]

Study of Immunochemical Recognition. In order to determine the recognition of the different synthetic structures by specific IgE antibodies, competitive inhibition immunoassays were carried out with those sera that showed positive results in the RAST, higher than 7%, using in the solid phase the culprit cephalosporin conjugated to PLL, as described.[34] The RAST inhibition test was undertaken using in the fluid phase the sera from the patients incubated with 10-fold concentrations (from 100 to 10 mM) of the compounds synthesized with different

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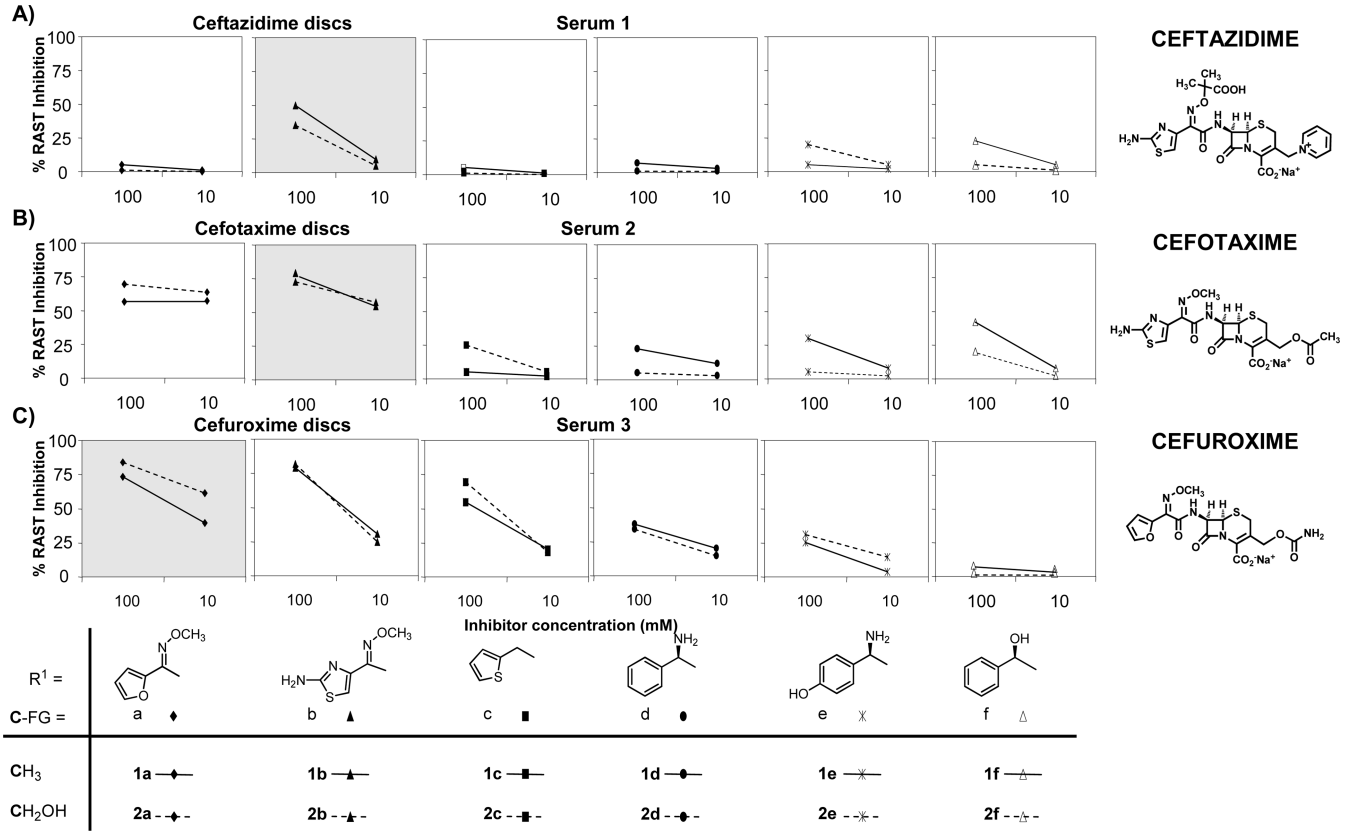

Figure 2. Comparison of the recognition of structures **1** (**a**−**f**) and **2** (**a**−**f**) by RAST inhibition studies in sera from patients allergic to ceftazidime (serum 1) (A), cefotaxime (serum 2) (B), and cefuroxime (serum 3) (C).

functionalities (H, OH, SH, and $O(CH_3)_2$) at the C-3 position in each case with six side chains at the R^1 position (Figure 1). Only two concentrations were used in order to save sera since many determinations were required because of the number of compounds to be tested. On the basis of previous studies, these concentrations, although high, were known not to induce nonspecific binding.[34] After 18 h of incubation at room temperature, discs sensitized with the culprit cephalosporin conjugated to PLL were added. Results were expressed as percentage inhibition with respect to the noninhibited serum.

RESULTS

Synthesis. To synthesize the series of proposed antigenic determinant structures, we followed the general retrosynthetic analysis described in Figure 1. The first disconnection of the amide bond leads to two synthons, the acid functional side chain, and the amine functional nuclear structure. The amide bond of the latter structure can be disconnected into a protected α-amino acid and *n*-butylamine. Therefore, the suggested antigenic determinant **1**−**3** (**a**−**f**) structures are proposed to be synthesized from the corresponding R^1 acyl side chain, plus a derived α-amino acid, and *n*-butylamine (Figure 1). In addition, the possibility of using different amino acids makes the synthetic methodology versatile, leading to different functional groups (FG) present in the nuclear structure fragment. The most basic structure involving our proposed antigenic determinant model (**1**, FG = H) presents a methyl group in the carbon corresponding to the 6-position remaining from the cephalosporins. The straightforward synthesis was carried out starting from an alanine derivative and the different R^1 acyl side chains.

Following the same methodology for model structures type **2** (FG = OH) and **3** (FG = SH), the nuclear structure fragments could be derived from serine and cysteine, respectively. Serine was used without any protection in the hydroxyl group and the use of cystin, instead of cystein, provided a protected thiol group during synthetic procedures. Both amino protected amino acids, *N*-Boc-L-serine and *N*-Boc-L-cystin, were reacted with *n*-butylamine via carboxyl group activation as a mixed anhydride, to obtain amides **6** and **9**, respectively (Scheme 3A). Their subsequent amine deprotection under acidic conditions yielded the nuclear structure fragments **7** and **10**, with an overall good yield.

To obtain the new third nuclear structure fragment, necessary to have model structures with a higher oxidation state at C-3 in the model structure, compounds **4** (FG = $(OCH_3)_2$), the amine nuclear structure fragment **15** was quantitatively obtained following an analogous methodology used for the synthesis of similar products,[30] described in Scheme 3B. The methoxy acrylate **11** was converted to bromo acetal **12** with *N*-bromosuccinimide (NBS)/MeOH by a bromo etherification reaction of the alkene.[30] Reaction of **12** with an excess of *n*-butylamine at room temperature selectively afforded bromo amide **13**, and the nucleophilic displacement of bromide **13** took place with an excess of benzyl amine at higher temperatures to give product **14**. The last step of hydrogenolysis with a Pearlman catalyst afforded the free primary amine **15**.

Amino-functionalized R^1 side chains (**b**,**d**,**e**) were employed as protected, $R^{1'}$, with either the *tert*butoxicarbonyl or the triphenylcarbinol group **16**(**b**,**d**,**e**). A nonreported synthesis and isolation of the pure Z isomer **16a** acyl side chain, which involved a

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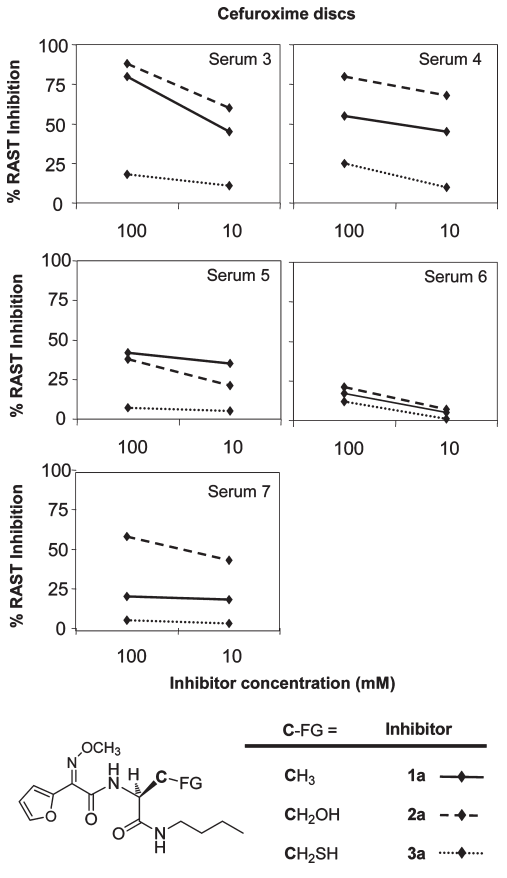

Figure 3. RAST inhibition studies in sera from five patients allergic to cefuroxime using compounds **1a** (FG = H), **2a** (FG = OH), and **3a** (FG = SH), at two concentrations (100 and 10 mM).

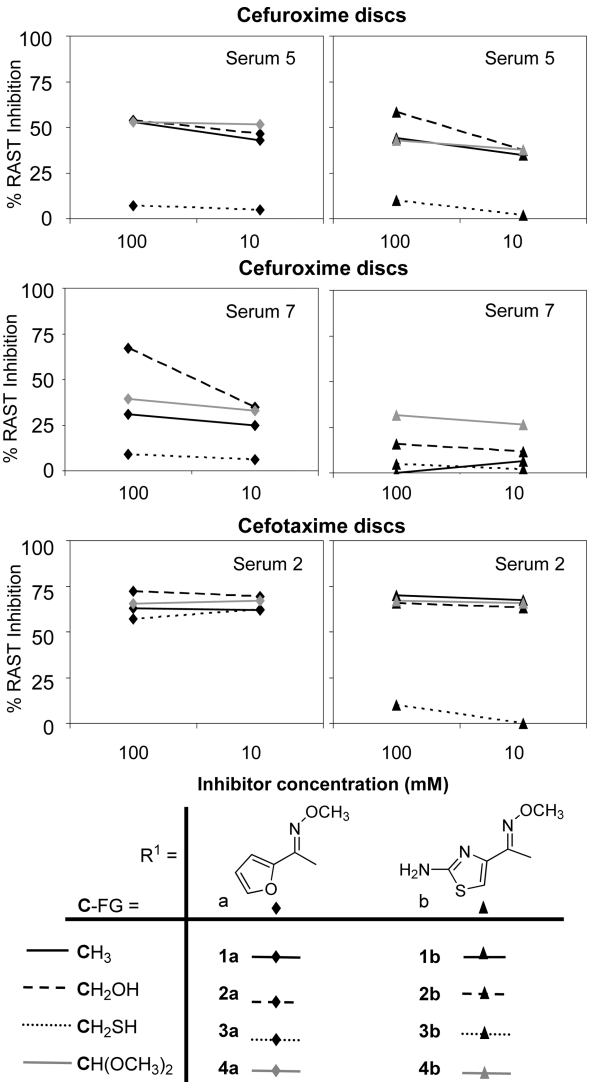

Figure 4. RAST inhibition studies in sera from three patients allergic to cephaloporins using compounds **1** (**a**,**b**) (FG = H), **2** (**a**,**b**) (FG = OH), **3** (**a**,**b**) (FG = SH), and **4** (**a**,**b**) (FG = $(OCH_3)_2$) at two concentrations (100 and 10 mM).

dehydrating condensation for imine formation and further recrystallization of the Z isomer in benzene, facilitated the preparation of structures **1a**−**4a**, avoiding the extensive chromatographic separations needed when the methoximine group was formed in the last step of the synthesis and therefore yielding higher overall conversions. The configuration of the **16a** Z isomer was determined by nuclear Overhouser effect spectroscopy (NOESY) experiments.

The three amine nuclear structure fragments (**7**, **10**, and **15**) were reacted with the acyl side chains **16a**−**f** via a previous carboxyl group activation with a carbodiimide reagent (Scheme 3C). An additional deprotection step under acidic conditions was carried out to molecules containing **b**, **d**, and **e** side chains. Furthermore, to obtain structures **3**, the disulfide bonds of symmetrical disulfides were finally reduced with DTT,[35] to liberate two equivalents of the desired thiols **3a**−**f**. The addition of TEA in this reaction to reach high pH was not necessary for the synthesis of products **3d** and **3e**.

All the products obtained, as well as the intermediates in their synthesis, were conveniently purified and analyzed to confirm the structure of the proposed molecules (Supporting Information).

Immunochemical Studies. The ability of IgE in sera from patients allergic to selected cephalosporins to recognize compounds **1**−**4** (**a**−**f**) was studied by competitive RAST inhibition immunoassays.

To study the effect of presenting a hydroxyl functional group at C-3 in model structures, containing the six R^1 side chains, on specific antibody recognition, the inhibition of a new synthesized series of compounds **2a**−**f** was compared to those involving the more basic structures **1a**−**f**. For this, three sera from patients with allergic reactions to ceftazidime, cefotaxime, and cefuroxime, respectively, were analyzed. Sera 1 (Figure 2A) and sera 2 (Figure 2B) achieved the maximal inhibition percentage with compounds **1b** and **2b**, which contain the same R^1 side chain (**b**) as the cephalosporins involved in the reaction. Serum 2 also showed a high recognition with compounds **1a** and **2a**, the side chain chemical structure of which is similar to that in the cephalosporin involved in the reaction. Serum 3 (Figure 2C), specific to cefuroxime, showed a maximal inhibition percentage with compounds **1a** and **2a**, which contain the same side chain as cefuroxime. In addition, there was an important specific binding to **1b**, **2b**, **1c**, and **2c**, showing as a consequence a high cross-reactivity to cephalosporins including a 5-membered heterocyclic structure in their R^1 side chain.

Regarding the comparison between different functional groups at C-3, (FG = H, OH), RAST inhibition studies showed

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Table 2. RAST Results with Different Cephalosporins[a]

		RAST								
case	drug involved	cefuroxime	cefotaxime	cefozidime	ceftriaxone	cefepime	cefonicid	cefaclor	ceftazidime	cefadroxil
1	ceftazidime	1.72	**3.09**	**2.02**	**2.27**	1.99	0.43	0.46	**23.31**	0.35
2	cefotaxime	**4.52**	**19.75**	0.35	**2.62**	**13.34**	0.49	1.03	0.25	0.11
3	cefuroxime	**9.43**	0.89	0.15	0.16	0.11	0.18	0	0	0
4	cefuroxime	**20.76**	**13.9**	**2.22**	1.99	1.51	1.08	0.73	0.67	0.03
5	cefuroxime	**2.61**	0.53	0.12	0.2	0.36	0.3	0.4	0.28	0.02
6	cefuroxime	**3.46**	0.18	0	0.29	0	0.29	0.06	0.15	0.14
7	cefuroxime	**13.15**	1.45	0.89	1.02	0	0.45	0	1.36	0.02

[a] Bold faced values indicate positive results.

the same tendency in the IgE recognition of either the methyl group in structures **1** or the hydroxymethyl group in structures **2**, with the chemical structure in the R^1 position being critical (Figure 2A−C). The extension of the study including the functional group thiol (Figure 3) showed a specific recognition of **1a** and **2a** molecules, with the better recognized in most cases being those with hydroxyl group **2a**, whereas there was poor recognition for the molecule with thiol group **3a**.

To complete the study, structures presenting two R^1 side chains, **a** and **b**, corresponding to cefuroxime and cefotaxime respectively, and four different functional groups (FG = H, OH, SH, and $(OCH_3)_2$) were evaluated through inhibition studies by using three sera from patients allergic to cephalosporins (Figure 4). Data showed that serum 5, specific to cefuroxime, showed higher recognition with three of the structures containing the cefuroxime side chain (**a**) and was similar with structures **1a**, **2a**, and **4a**. The same serum also showed inhibition with compounds containing the cefotaxime side chain (**b**), again with similar recognition of **1b**, **2b**, and **4b**. There was no recognition of compounds containing the thiol functional group at C-3, **3a** and **3b**. For serum 7, also specific to cefuroxime, the RAST inhibition assay showed a strong binding with structure **2a**, followed by **4a** and **1a**, with no recognition of **3a**. Among compounds containing the cefotaxime side chain, only structure **4d** showed a weak recognition. Serum 2, specific to cefotaxime, showed a specific binding with the structures **1b**, **2b**, and **4b**, and no inhibition with **3b**. This sera also showed high inhibition with compounds (**1**−**4**)**a**, which included the cefuroxime side chain.

No recognition was obtained with the compounds **1**−**4** (**d**−**e**) no matter the group present at C-3. This is because these compounds have the side chain **d** of cefaclor or cephalexin, **e** of cefadroxil or cefprozilo, and **f** of cefonicid or cefamandole. Significantly, the different sera included in the study came from patients allergic to cephalosporins in which the culprit cephalosporin was ceftazidime, cefotaxime, or cefuroxime, which have a very different side chain at R^1 position.

All these patterns of recognition of different compounds depending on the R^1 side chain correlated with the skin test and direct RAST results (Table 2).

DISCUSSION

Despite the uncertainty about structures comprising the epitopes of cephalosporins, the same clinical behavior model has been assumed for cephalosporins and penicillins, without considering the different chemical reactivities of both β-lactams.[27,36] Consequently, clinicians have to make difficult decisions in order to select β-lactams for penicillin/cephalosporin allergic patients since many questions about cross-reactivities between cephalosporins and between cephalosporins and penicillins are still unresolved. The lack of standard in vitro diagnostic methods has led to the use of in vivo methods using the native cephalosporin, which can be risky. Therefore, the consumption of these β-lactams is unsafe when the subject has had an adverse reaction to just one β-lactam.

The results showed that the specificity of IgE antibodies from all the patients evaluated recognized these synthetic structures with different patterns that correlated with the in vivo and in vitro patterns of response reported in previous studies.[17,28] To summarize the overall trend seen in most sera, the cases showed optimal inhibition with some of the proposed model structures containing the R^1 side chain of the cephalosporin which induced the reaction. Additionally, different degrees of cross-reactivity were observed with structures containing the R^1 side chain with a similar chemical structure. This is in agreement with previous studies where IgE recognition was evaluated with other approaches, such as skin test or RAST.[17,18]

Regarding the influence of the functionality at C-3, RAST inhibition studies showed similar IgE recognition to the proposed model structures when the C-3 formed part of a methyl (**1**), hydroxymethyl (**2**), or dimethoxyacetal group (**4**). Furthermore, results indicated that molecules containing FG = OH and FG = $(O(CH_3)_2)$ at C-3, structures **2** and **4**, respectively, slightly enhanced the IgE recognition in most cases (sera 3−7). This improvement in the IgE molecular interaction process indicates that these structures mimic the real antigenic determinant involved in the initial immunological recognition. The actual antigenic determinants, as a consequence, likely contain a higher oxidation state than that corresponding to a methyl group at C-3 in the model structure, which is in agreement with the fact that the C-6 in the native cephalosporin presented an oxidation state equivalent to a carbonyl functional group and therefore higher than that corresponding to the methyl group.

However, despite the fact that the thiol group provided C-3 with an oxidation grade equivalent to the hydroxyl group, the simple presence of a sulfur atom on structures **3** drastically decreased the inhibition and showed no inhibition in most cases. The lack of IgE recognition obtained with structures **3**, even though they contained the same R^1 side chain as the cephalosporin inducing the reaction, provides significant information regarding the nuclear structure fragment of the antigenic determinant. Thus, the sulfur atom may not remain bonded to the epitope.

These data help understand the generation of metabolites which are later recognized by specific IgE, particularly relating to

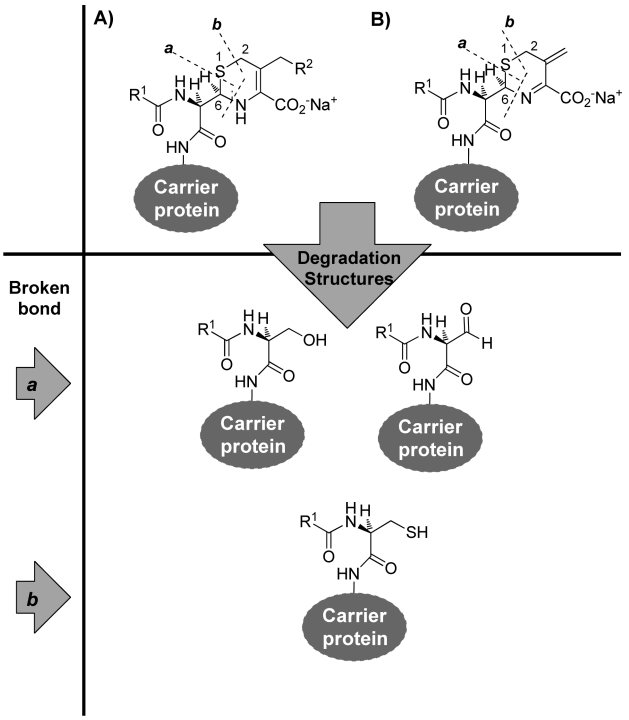

Figure 5. Hypothetic degradation structures derived from cephalosporin conjugation depending on the broken bond. (A) Nonconcerted process intermediate; (B) structure which eliminated its R^2.

the broken bond in the cephalosporins during the formation of their antigenic determinant. The hypothetical intermediate structures after conjugation of the cephalosporin to proteins are described in Figure 5, indicating the bonds which could undergo fragmentation. Since model structures **2** and **4**, involving hydroxymethyl and acetal groups as C-3, were the best recognized by specific IgE, the in vivo formation of cephalosporin antigenic determinants could occur via fragmentation of the S1−C6 bond.

These new well-defined structures help us understand epitope formation after conjugation of the cephalosporin to carrier proteins. The findings confirmed that besides recognition at the R^1 acyl side chain structure, the rest of the nuclear structure is also important for optimal molecular recognition.

Several toxicological effects can result from the covalent binding of small drug molecules to proteins.[37] Structural studies of cephalosporin−protein adducts have never successfully been addressed and are difficult to investigate. The way in which the drug metabolizes after protein conjugation is very important not only from a chemical point of view but also from an applications in the clinical diagnosis of allergy point of view. The use of well-defined structures and their exact concentrations in these immunoassays provide precise information about the chemical requirements of IgE recognition to cephalosporins. Whether a monomeric conjugate permits a better or worse inhibition and thus different recognition by specific IgE antibodies provides information about the relevant role of the different parts of the cephalosporin molecule at the hapten binding site and, therefore, about the nature of the recognition process and epitope composition. As a rule, evaluated sera had optimal recognition with structures containing the same R^1 side chain as the cephalosporin inducing the allergic reaction and, in cross-reactivity cases, structures including a similar R^1 structure. The functional groups tested in C-3, hydroxymethyl, and acetal groups showed the same pattern with improved inhibition compared to the methyl group. From a practical point of view, structures **2** (FG = OH) have an additional advantage when included in the test since they present higher solubility in aqueous media. As these structures can be detected with a high specificity, model structures **2** could be useful in the development of in vitro methods for detection and quantification of IgE specific to cephalosporins.

CONCLUSIONS

A series of well-defined synthetic chemical structures proved to be elegant tools to investigate the antigenic determinants to cephalosporin, which is otherwise difficult to study. The proposed epitopes, **1**−**4** (**a**−**f**), consist of closely related low-molecular-weight molecules, differing only in the functionality at position C-3 and the R^1 side chains. Fine structural selective recognition was detected between IgE from patients allergic to cephalosporins and our proposed skeleton epitopes involving the appropriate functionality and R^1 side chain. Thus, as a result of this research we have been able to refine the earlier design of model antigenic determinants which fulfill the structural implications needed for recognition by IgE antibodies to cephalosporin and which are consequently responsible for the adverse reactions. Besides the improvement obtained with the new structures (**2** and **4**) in IgE molecular recognition, the aqueous solubility benefit of hydroxyl functionalized model structures (**2**) makes them potential candidates for a useful in vitro diagnostic test. We have shown here that minor alterations of the structure can result in major deviations in the ability to be recognized by the IgEs. As a result, the change of the oxygen atom in model compounds **2** by a sulfur atom, such as compounds **3**, as can be supposed from the hypothetical pathways of fragmentation of the starting antibiotic, produces a significant diminution in the possibility of being recognized by IgEs. Other chemical modifications of the model structures, as well as their conjugation to carrier proteins and solid phases, are under way to extend the evaluation of structural implications in the recognition process and develop clinically useful diagnostic tests.

ASSOCIATED CONTENT

Supporting Information. Structural characterization including MS, ^1H, and ^{13}C NMR are described for final structures **2**−**4** (**a**−**f**) and intermediate pure products needed for the synthesis. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Phone: (+34) 952137565. Fax: (+34) 952131941. E-mail: inestrosa@uma.es.

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DEDICATION

This article is dedicated to Professor Rafael Suau, who passed away before the submission of this article. Dr. Suau was an

eminent scientist and a good friend, and he was highly involved in this research.

■ ABBREVIATIONS

CPO, cephalosporoyl; DCC, *N*,*N*′-dicyclohexylcarbodiimide; FG, functional group; EDCI, 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride; ENDA, European Network of Drug Allergy; HOBt, *N*-hydroxybenzotriazole; MDM, minor determinant mixture; NBS, *N*-bromosuccinimide; NMM, *N*-methylmorpholine; NOESY, nuclear Overhouser effect spectroscopy; PLL, poly-L-lysine; PO, penicilloyl; RAST, radioallergosorbent test; 2 SD, standard deviation; TEA, triethylamine.

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