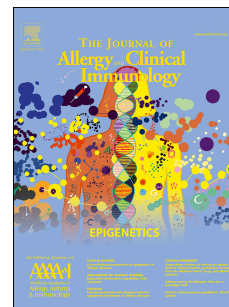


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Design of an antigenic determinant of cefaclor: Chemical structure–IgE recognition relationship.

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Conflicts of interest: none

Keywords

Allergy, betalactam, cephalosporin, diagnosis, drug, epitope, IgE, *in vitro* test, pyrazin-2(1*H*)-one, Ugi/Deprotect/Cyclize strategy.

Abbreviations

IgE, immunoglobulin E; PLL, Poly-L-Lysine; RAST, RadioAllergoSorbent Test; SAR, Structure-Activity Relationship; UDC, Ugi/Deprotect/Cyclize.

total word count: 990

Capsule summary:

A novel pyrazinone epitope of cefaclor has been synthesized, which is specifically recognized by IgE from patients allergic to cefaclor, shedding light into the mechanisms involved in allergy to α -aminocephalosporins.

To the Editor:

Like penicillins, cephalosporins are common antibiotics inducing adverse drug reactions mediated by specific immunological mechanisms.¹ The "hapten hypothesis" states that β -lactams can only cause an allergic response after binding covalently to carrier proteins. In case of cephalosporins, the epitope or antigenic determinant consists of the chemical structure derived after cephalosporin-protein linkage. The lack of knowledge regarding its epitope has hampered the development of standardized *in vitro* tests to diagnose cephalosporin allergy. Therefore, diagnosis still relies on skin testing and drug provocation tests;² which, however, are risky, especially for patients with severe reactions. This forces physicians to select alternative β -lactams that, although safe for allergic patients, may be less effective and show disadvantages related to costs and antibacterial-resistance.³ The current *in vitro* tests (immunoassays and basophil activation test) although complementary to *in vivo* tests, do not have optimal sensitivity. To improve the sensitivity of *in vitro* tests it is necessary to consider the epitope chemical structures and their immunological recognition, for which we need to better understand the drug degradation pathways.³

Cephalosporin conjugation to proteins results in the cephalosporoyl formation through an amide bond (Fig 1, A), whose highly unstable structure (Fig 1, B) generates different degradation products that have not been identified, preventing us from understanding the cephalosporin-immunogenicity mechanisms.¹ There is clinical and immunochemical evidence of R¹ side chain contribution to specific IgE recognition: some patients are selective to the culprit cephalosporin, whilst others cross-react to other cephalosporins,^{4,5} or other β -lactams.⁵ To our knowledge, Structural Activity Relationship (SAR) studies have been the unique successful approach for investigating cephalosporins epitopes.^{1,4,6-8} We have elucidated precise epitope structures through the synthesis and immunological evaluation of well-defined structures proposed as antigenic determinants, bearing different functionalities at the C-6 of the cephalosporin (**1**, methyl; **2**, hydroxymethyl; **3**, acetal; **4**, mercaptomethyl) (Fig 1, A).⁶⁻⁸ Those with hydroxyl (**2**) or aldehyde (**3**) functionality have been proven to show the strongest immunological recognition for most cephalosporins.^{6,7} However, a different pattern, with less recognition of these structures was observed for α -aminocephalosporin allergic patients, suggesting the existence of other determinants. This could be explained by the additional reactivity due to the amino group in the R¹ side chain, which may react with the likely aldehyde functionality at C-6 of structure **3**,⁷ leading to intramolecular cyclization followed by oxidation (Fig 1, B). This process results in a pyrazinone-like derivative **5** isolated for cefaclor and cephalexin, although in very low yields. A synthetic pyrazinone analogue, structurally different from the isolated molecule, showed recognition in cefaclor allergic patients.⁸

Here, to better define the structural immunoreactivity of cefaclor, we performed a SAR study of the cyclized structure (**5**) as potential antigenic determinant. For this, an efficient synthetic methodology of the novel determinant was developed, and its immunological recognition was evaluated using sera from cefaclor allergic patients.

The pyrazinone determinant was designed according to cefaclor degradation hypothesis, using butylamine as a model nucleophile emulating lysine (Fig 1, B). The straightforward synthesis of pyrazin-2(1*H*)-one (**5**) was achieved utilizing the Ugi/Deprotect/Cyclize strategy (UDC) (Fig 1, C) with quantitative yield.⁹ First, required structures were assembled following the one-pot Ugi four-component reaction, in which butyl isocyanide, a protected amine (2,4-dimethoxybenzylamine), a protected aldehyde (2,2-dimethoxyacetaldehyde) and a carboxylic acid (N-Boc phenylglycine) yielded the Ugi adduct. Subsequently, acid treatment may result in the amino-functionalized aldehyde intermediate, which, after cyclization through intramolecular imine formation and aromatization, directly afforded target pyrazinone. This was a relatively simple method as well as time- and cost-effective compared to other synthetic alternatives, involving multi-step purification procedures or unsuccessful intramolecular cyclization.⁹ For details of synthesis and characterization, see Methods section and Fig E1 in this article's Online Repository at www.jacionline.org.

The ability of sIgE in sera from cefaclor allergic patients to recognize the pyrazinone **5** was evaluated by competitive inhibition of RadioAllergoSorbent test (RAST) and compared with the previously reported structure **2**. This assay consists in competitive IgE recognition between the solid phase (poly-L-lysine-drug conjugate attached to cellulose) and the inhibitors (**5** and **2**) at different concentrations in the fluid phase. For details of *in vitro* immunoassays and antibody radiolabeling, see Methods section in this article's Online Repository at www.jacionline.org.

We selected a group of 8 patients with immediate allergic reactions to cefaclor, whose diagnostic evaluation consisted in positive skin testing and presence of sIgE antibodies against cefaclor in sera (see Methods section and Table E1 and Table E2 in this article's Online Repository at www.jacionline.org). The evaluation of both determinants in sera from selected patients (Figure 2), by RAST inhibition, showed in most cases, at the maximum concentration (100 mM) of determinants, a higher percentage of inhibition for the pyrazinone **5** than for the determinant **2**, indicating a better recognition of the cyclized structure. In general, inhibition dropped at the minimum concentration (10 mM) of inhibitor. *In vitro* IgE recognition is generally considered meaningful when the inhibition percentage is higher than 50%. Although statistically significant differences in the percentage of inhibition were not observed, differences in the positivity were remarkable, since 63% of cefaclor allergic patients recognized the pyrazinone **5** whereas only 12% of cases recognized the determinant **2**, at the maximum concentration. Interestingly, the percentage of patients showing recognition to any of the structures increased to 75% when results obtained with determinants **5** and **2** were combined.

This is the first time that the precise cyclized structure **5** has been synthesized and immunologically evaluated, showing that it mimics better the real antigenic determinant than the previously reported structure **2**. Despite the population size limitation, an outstanding IgE recognition was obtained with pyrazinone **5** alone; however, we believe that other structures can also be generated. Indeed, the inclusion of the different synthetic structures would be recommended for diagnosing the maximal number of patients, ensuring the detection of different patterns of recognition. Moreover, after the

promising results in *in vitro* study, the evaluation of molecule **5** on skin testing in allergic patients should be helpful to further understand *in vivo* significance.

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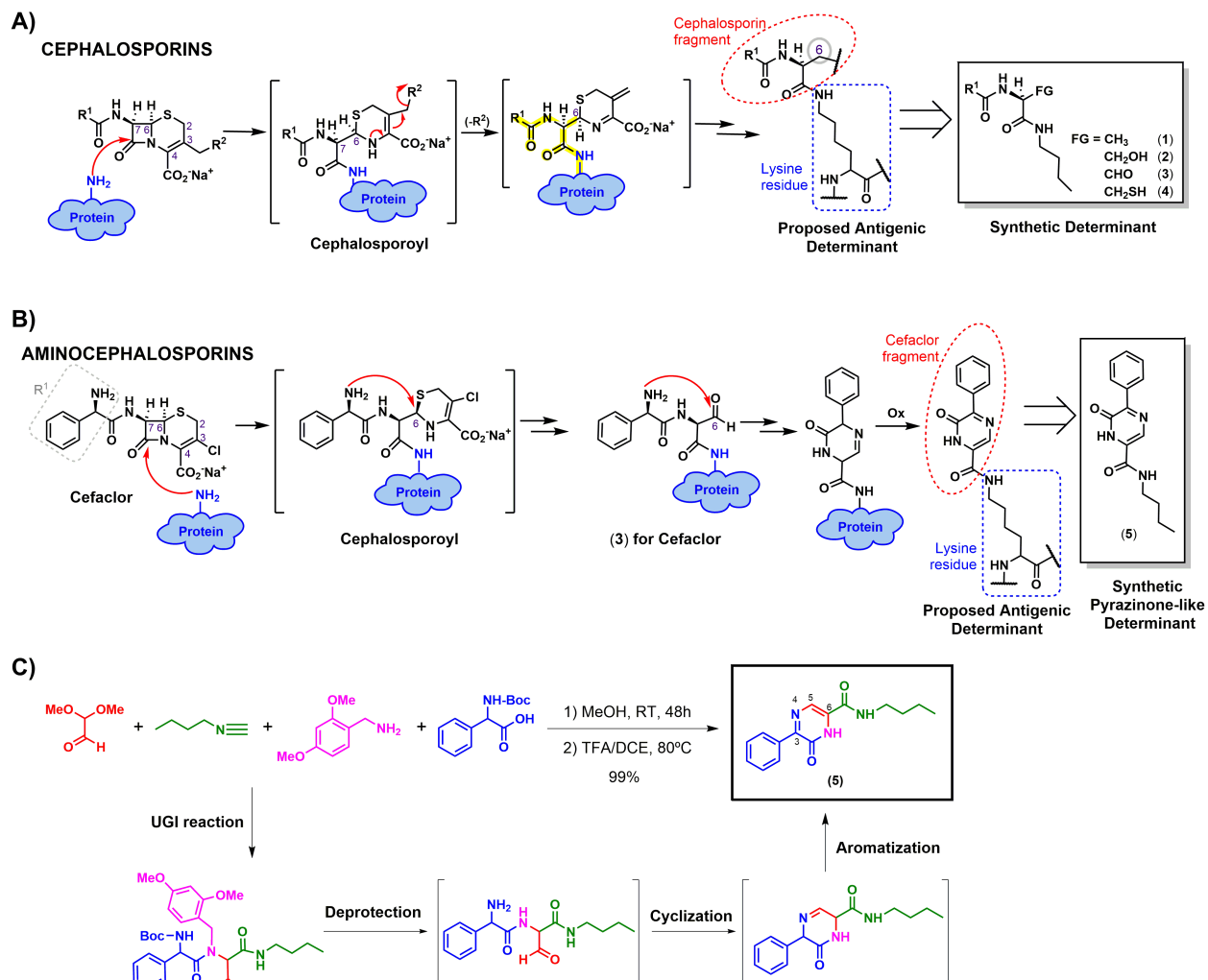
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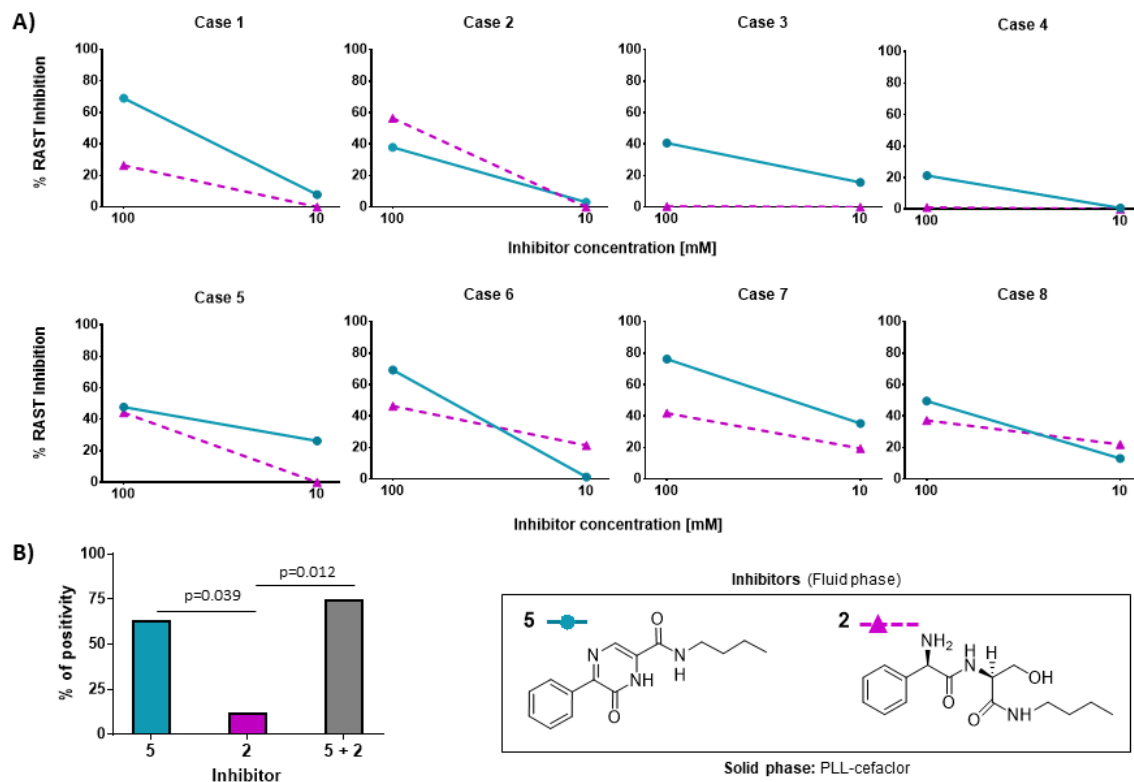
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FIGURE LEGENDS

Figure 1. **A**, Degradation hypothesis of cephalosporins after nucleophilic opening of β -lactam ring by protein amino groups, leading to the cephalosporoyl intermediate and eventually resulting in the proposed antigenic determinants. In the square are represented those structures that have been previously synthesized and immunologically evaluated. **B**, Degradation pathway hypothesis of cefaclor after protein conjugation, in which the aldehyde **3** is a key intermediate in the formation of pyrazinone **5**, through intramolecular imine bonding and subsequent aromatization. **C**, Synthesis of pyrazin-2(1*H*)-one, as proposed cefaclor epitope, by means of Ugi/Deprotect/Cyclize strategy (UDC) strategy.

Figure 2. Immunological evaluation results with cefaclor derivatives. **A**, RAST inhibition assays performed with sera from 8 patients allergic to cefaclor, using compounds **5** and **2** as inhibitors and cellulose discs modified with cefaclor-PLL as the solid phase. **B**, Comparisons among both inhibitors (**5** and **2**) and the combination of both results (**5+2**), in percentages, of positive inhibition cases at 100 mM concentration. Significant differences (calculated using Chi-square test) are indicated in the graph.





Case	Age	Sex	Reaction	Grade Severity ^a	Drug involved in the reaction	INT (days)	Skin test																	
							BP-OL		MD		AX		AMP		Cefaclor (culprit)		Cephalexin		Cefadroxil		Ceftriaxone		Cefuroxime	
							SPT	IDT	SPT	IDT	SPT	IDT	SPT	IDT	SPT	IDT	SPT	IDT	SPT	IDT	SPT	IDT	SPT	IDT
1	38	M	Urticaria	I-Mild	Cefaclor	7	-	-	-	-	-	-	-	-	-	+	ND	ND	ND	ND	ND	ND	ND	ND
2	43	F	Urticaria and AE	I-Mild	Cefaclor	NK	-	-	-	-	-	-	-	+	-	+	ND	ND	ND	ND	ND	ND	ND	ND
3	37	F	Anaphylactic shock	III-Severe	Cefaclor	240	-	ND	-	ND	-	ND	-	ND	+	ND	ND	ND	ND	ND	ND	ND	ND	ND
4	56	F	Anaphylaxis	II-Moderate	Cefaclor	120	ND	ND	ND	ND	ND	ND	-	-	+	ND	ND	ND	-	-	-	-	-	-
5	13	F	Anaphylactic shock	III-Severe	Cefaclor	450	-	-	-	-	-	+	-	+	+	ND	-	+	ND	ND	-	-	-	+
6	5	F	Generalized erythema	I-Mild	Cefaclor	30	-	-	-	-	-	-	-	-	-	+	-	-	ND	ND	-	-	-	-
7	17	F	Anaphylaxis	III-Severe	Cefaclor	30	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-
8	27	F	Urticaria and AE	I-Mild	Cefaclor	210	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-

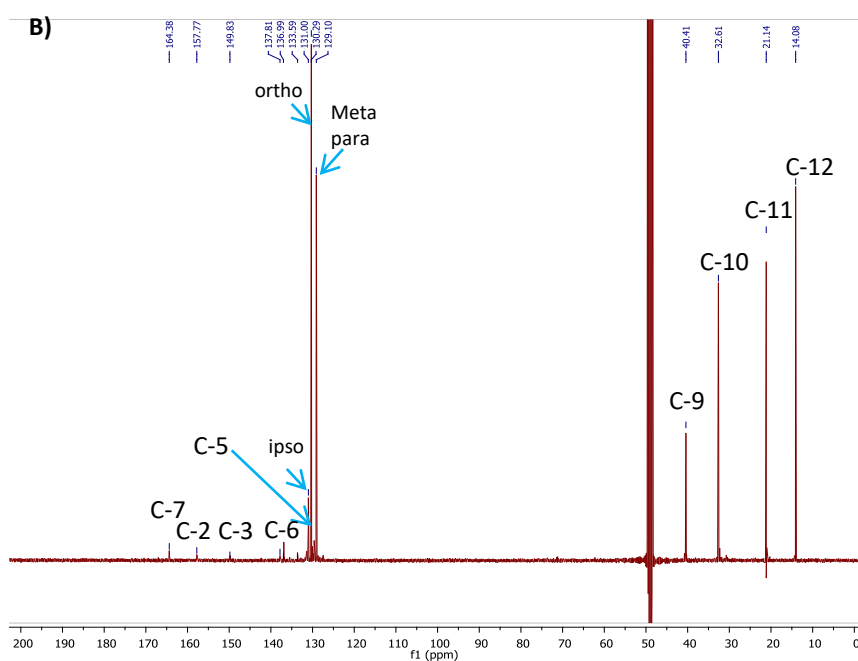
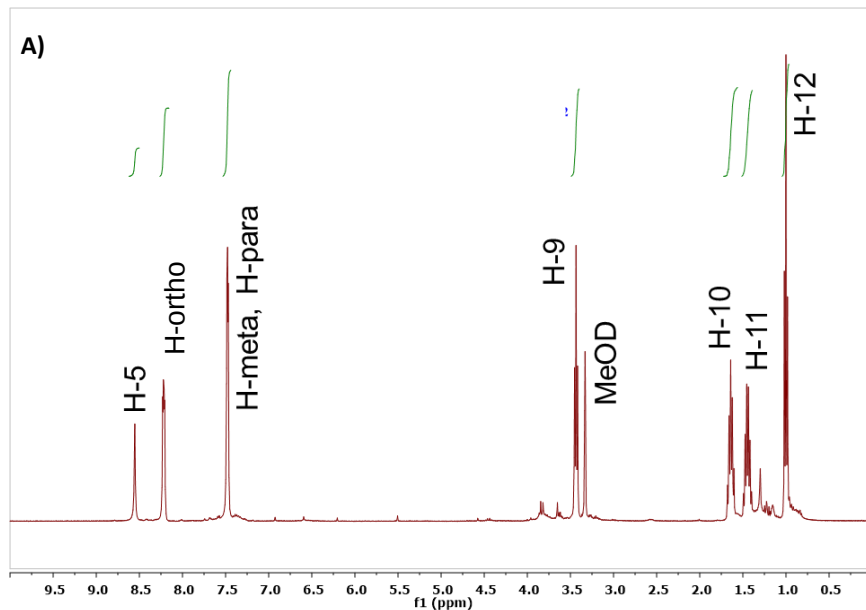
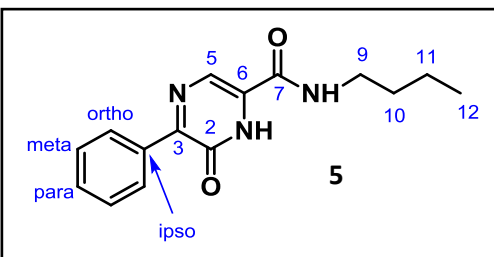
Table E1. Clinical data and results of *in vivo* test of patients diagnosed with an immediate allergic reaction to cefaclor included in the study. F: female; M: male; AE: angioedema; INT: time interval between reaction and study; (+): positive; (-): negative; ND: not determined; SPT: skin prick test; IDT: intradermal test; BP-OL: benzylpenicilloyl-octalysine; MD: minor determinant.

^aGrading system for generalized hypersensitivity reactions: I Mild (skin and subcutaneous issues only); II Moderate (features suggesting respiratory, cardiovascular, or gastrointestinal involvement); III Severe (hypoxia, hypotension, or neurologic compromise) (Brown SG. *J Allergy Clin Immunol.* 2004 Aug;114(2):371-6).

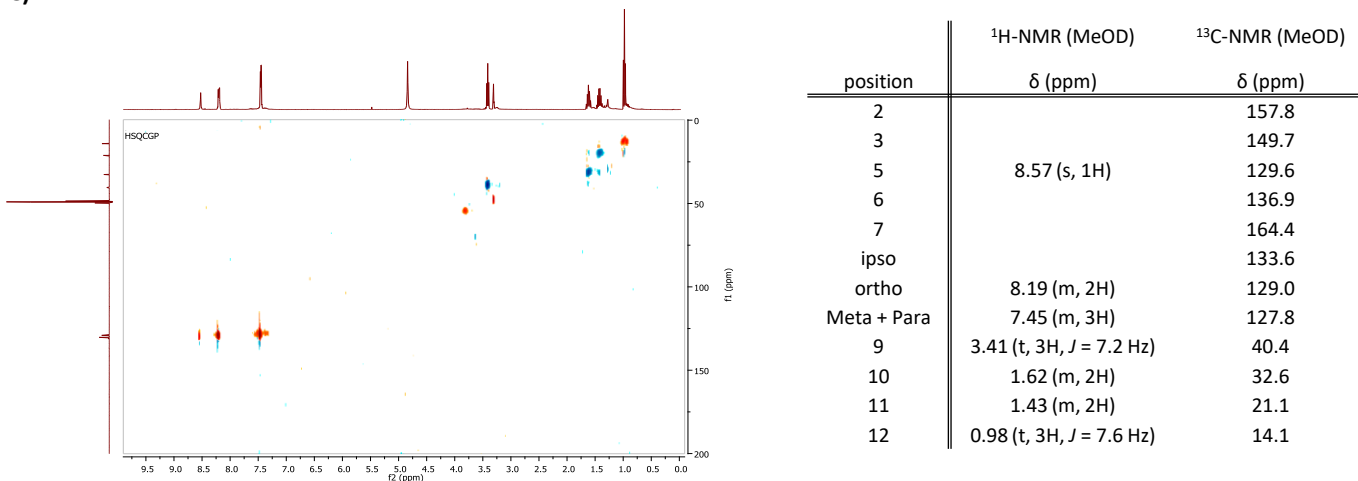
Case	% RAST ^a	ImmunoCAP Cefaclor ^a	
	Cefaclor-PLL	Specific IgE (kUA/l)	Reaction class
1	33.37	4.74	3
2	15.36	0.64	1
3	20.55	3.22	2
4	19.44	8.95	4
5	11.17	0.12	0
6	34.56	0.02	0
7	7.69	1.17	2
8	11.67	2.53	2

^aBold faced values indicate positive results

Table E2. Results of *in vitro* test of patients diagnosed with an immediate allergic reaction to cefaclor included in the study. PLL: poly-L-Lysine; RAST: radioallergosorbent test.



c)



METHODS

Synthesis of N-butyl-6-oxo-5-phenyl-1,6-dihydropyrazine-2-carboxamide (5)

Ugi/Desprotect/Cyclize (UDC) strategy was employed for synthesizing the desired product.^{E1} Equimolar amounts of each substrate (butyl isocyanide, 2,5-dimethoxybenzylamine, 2,2-dimethoxyacetaldehyde solution 60% in H₂O and N-Boc-phenylglycine) were left to react in methanol (MeOH) at room temperature until Ugi adduct formation was complete (thin layer chromatography evidence). The time needed for Ugi adduct formation was 7 days. To remove possible remaining isocyanide, dichloromethane (DCM) and polystyrene-supported p-toluenesulfonic acid (PS-p-TsOH) were added and submitted to orbital stirring at room temperature for approximately 1.5 hours. The PS-p-TsOH was filtered off and successively washed with MeOH, ethyl acetate (EtOAc) and DCM (3 times with 5 mL of each solvent). To deprotect and cyclize, the residue obtained after solvent evaporation (Ugi adduct) was treated with 30% trifluoroacetic acid (TFA) in dichloroethane (DCE) and heated at 80°C for 2 hours. The solution was then treated with a saturated solution of NaHCO₃ until pH 8 and extracted with EtOAc. The organic phase was dried over MgSO₄ and evaporated under reduced pressure to produce an oily yellow residue that was purified by chromatographic methods on silica gel using DCM/MeOH mixtures, with the addition of 2% NH₃ to obtain high recovery. Fair yellow solid, 99% yield. Melting point= 172-176°C. [α]_D= -21 (c₁= 0.2, MeOH). Nuclear magnetic resonance (NMR) spectra of the pure compound are showed in the Figure E1. ¹H-NMR (400 MHz, MeOD): δ (ppm) 8.52 (s, 1H; N-CH=CCO), 8.19 (m, 2H; Aromatic-ortho), 7.45 (m, 3H; Aromatic-meta+para), 3.41 (t, ³J(H,H) = 7.2 Hz, 2H; -CONH-CH₂-), 1.62 (m, 2H; -CH₂-CH₂-CH₂-), 1.43 (m, 2H; -CH₂-CH₂-CH₃), 0.98 (t, ³J(H,H) = 7.6 Hz, 3H; -CH₂-CH₃). ¹³C-NMR (100 MHz, MeOD): δ (ppm) 164.3, 157.7, 149.8, 136.9, 133.5, 131.0, 130.29, 129.1(x2), 40.4, 32.6, 21.1, 14.1. Electrospray ionization mass spectrum (ESI MS) calculated for C₁₅H₁₇N₃O₂ (M + H)⁺: 272.1399, found: 272.1393.

SUBJECTS

Patients selection

Patients with a clinical history of immediate allergy to cefaclor were evaluated following the European Network of Drug Allergy (ENDA) guidelines^{E2,3}. Those with a positive skin test to cefaclor were eligible for being included in the study. From these patients, we selected eight cases that showed high levels (greater than 7%) of specific IgE antibodies to cefaclor measured by direct Radio Allergo Sorbent Test (RAST). Clinical data from the patients included in the

study are included in Table E1, and *in vitro* evaluation by determination of specific IgE levels are shown in Table E2.

The study was conducted according to the Declaration of Helsinki principles and was approved by the Provincial Ethics Committee of Malaga. All subjects included in the study were informed orally and signed the corresponding informed consent.

Skin tests

Skin testing was performed by prick, and if negative followed by intradermal testing as recommended.^{E2, E3} The maximum concentrations used were as follows: BP-OL 0.04 mg/mL (with a concentration of the benzylpenicilloyl [BPO] moiety of 8.64×10^{-5} M), minor determinant (MD) 0.5 mg/mL (with a concentration of sodium benzylpenilloate of 1.5×10^{-3} M), amoxicillin 20 mg/mL (all from DIATER Laboratories, Madrid, Spain); ampicillin 20 mg/mL (Normon SA), and the following cephalosporins at 2 mg/mL: cefaclor (Lilly, Madrid, Spain), cefuroxime (GlaxoSmithKline S.A, Madrid, Spain), cephalexin (Keforal, Crinos S.p.A., Milan, Italy), cefadroxil (Genefarma, Valencia, Spain). In cases with a history of severe anaphylaxis we performed the intradermal test with lower concentrations (1/10 dilution), and if negative until the maximum concentration was reached.^{E4} In skin prick tests, a wheal larger than 3 mm with a negative response to the control saline was considered positive. In 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.

***In vitro* IgE Determination**

The serum sIgE levels were measured using two different methods: custom-made RAST and commercial ImmunoCAP. The *in vitro* IgE determination by RAST was made using cefaclor conjugated to Poly-L-Lysine (PLL) (Sigma, St. Louis, MO) in the cellulose solid phase as previously described.^{E5} Briefly, 30 μ L of patient sera was added over the solid phase functionalized with the corresponding PLL-drug conjugates and incubated for three hours. After washing, a radiolabelled pool of anti-human-IgE antibodies (kindly provided by Thermo Fisher Scientific and radiolabelled in our laboratory) was then added and incubated overnight. Finally, the solid phases were washed and radioactivity measured in a gamma-counter Cobra II autogamma (Packard BioScience Company, Frankfurt, Germany). Results were calculated as percentage of the maximum and considered positive if they were higher than 4.8%, which was the mean +2SD (standard deviation) of a negative control group. The sIgE levels were also

determined using the fluorescent enzyme immunoassay (ImmunoCAP-FEIA, Thermo Fisher Scientific Inc., Sweden) for cefaclor.

***In vitro* 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%). The assay consists in a competition of IgE recognition between solid phase (poly-L-lysine- β -lactam) and the inhibitors (**5** or **2**) in fluid phase. RAST inhibition test was undertaken using in the fluid phase the sera from the patients incubated with two 10-fold concentrations (100 and 10 mM) of the synthesized compounds. The two concentrations were chosen on the basis of previous studies which showed that these concentrations, although high, were known not to induce nonspecific binding.^{E6} After 18 h of incubation at room temperature, solid phase discs sensitized with the cefaclor conjugated to PLL were added, followed by previous procedure described for IgE determination. Results were calculated as percentage of inhibition with respect to the non-inhibited serum.

Antibody radiolabeling for detection in immunoassays

A mix of two anti-human-IgE monoclonal antibodies provided by Thermo Fisher Scientific was diluted at 1 mg/mL with PBS 1X (pH 7.4). An aliquot of 15 μ L (15 μ g) was separated from the stock solution and volume completed up to 45 μ L with PBS 1X. Previous mixture was added to the bottom of a Pierce Pre-Coated Iodination Tube (Thermo Fisher Scientific) and, immediately after, 2.4 μ L (250 μ Ci) of NaI¹²⁵ (NEZ033A001MC, PerkinElmer) 103, 23 mCi/mL concentration and 17 Ci/mg specific activity, incorporated to the reaction and left to react at room temperature for 15 minutes. After this time, the mixture was taken out of the iodination tube and the volume completed with PBS 1X up to 500 μ L to stop the reaction (stop solution). We kept 5 μ L to measure the maximum as reference for calculations. For antibody purification, a sephadex PD Minitrap G-25 (GE Healthcare Life Sciences) column was used. Fractions of 0.5 mL were collected and radioactivity measured using a HIDEX 300 SL gamma counter for identification of fractions containing radiolabelled IgG. Antibody containing fractions were combined and iodination yield, incorporated activity and specific activity were calculated.

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Figure Legends

FIG. E1. Nuclear Magnetic Resonance (NMR) characterization of structure **5**.

A) ^1H -NMR spectrum, **B)** ^{13}C -NMR spectrum, **C)** HSQC (Heteronuclear Single Quantum Coherence) experiment with gradient pulse. Bidimensional NMR spectrum (left) and signal assignation (right).