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Synthesis of skeletally diverse β-lactam haptens for the *in vitro* diagnosis of IgE-mediated drug allergy

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We present the first synthesis of β-lactam-derived haptens, leveraging the principles of diversity-oriented synthesis to discover compounds for drug allergy *in vitro* testing. We designed, synthesised, and performed in vitro immunological evaluation on 18 structurally diverse haptens derived from β-lactam antibiotics. The antigens obtained with the synthesised haptens allow for the detection of specific anti-β-lactam immunoglobulins G and E. Excellent diagnostic sensitivity (83%) and specificity (100%) were achieved when the panel of antigens was tested against a cohort of 31 human serum samples using a multiplexed compact disc-based in vitro testing tool. We posit that adopting this strategy could aid β-lactam delabeling initiatives.

Over 6.5% of the globally studied population carries penicillin or β-lactam allergy label.[1] The overdiagnosis of β-lactam allergy has several clinical consequences. Inpatients with such a label often receive second-line treatments, which are less effective, often more toxic, and associated with a higher risk of antibiotic-resistant infections. To reduce the risk of near-fatal reactions from in vivo allergy tests, the clinical guidelines advise that the in vivo allergy tests should be preceded by in vitro IgE tests for subjects with a history of severe immediate reactions.[2] However, current in vitro tests provide only a gross prediction of drug allergy, and negative results (< 0.35 IU/mL) do not necessarily exclude the possibility of β-lactam allergy, with the mean clinical sensitivity at only ~25%. Consequently, there is a need for additional reagents (haptens and antigens) to develop accurate in vitro assays to discriminate selectively and with high sensitivity allergic from non-allergic subjects.[3] Experimental studies on the synthesis of β-lactam-derived haptens are limited. Recent studies have described the synthesis of new haptens that incorporate spacers to create distance between the antibiotic molecule and the carrier protein, revealing that the inclusion of different drug-derived conjugates during sIgE evaluation can improve the accuracy of diagnosis.[4] Other study reports pyrazinone derivatives as novel antigenic determinants of α-aminocephalosporins showing a low percentage of positivity.[5] Indeed, to meet the challenge of the accumulated burden and increasing demand, allergy specialists need to be supported with innovative, evidence-based approaches for drug-allergy assessment and delabeling. In this respect, diversity-oriented synthesis (DOS) provides an appealing route to access compounds with structural complexity and diversity and incorporate chemical features common to natural products, such as sp3-hybridized carbon atoms and basic nitrogen atoms stereogenic elements, and novel skeletons.[6] To this aim, we synthesised using DOS a collection of 18 haptens using different β-lactam antibiotics containing greater structural diversity than typically observed for these antibiotics.

Using 6-aminopenicillanic acid (6-APA)—the precursor of all semi-synthetic penicillins—we performed a Petasis three-component, boronic-acid Mannich reaction followed by amine propargylation to yield β-amino alcohols, which were subjected to an array of skeletal diversification reactions.[7] Herein, we improve the yield of the three-component reaction involving 6-APA using different organic solvents and conditions (Table S1, ESI†). Using these improved conditions, we were with 6-APA methyl ester (**1a**) to gain access to compounds **4a**-**11a** (Scheme 1). The best conditions comprised a solvent system of 9:1 EtOH/HFIP (v/v) at 40 °C for 72 h, using an excess of both the antibiotic-based ester 1a and (E)-2-cyclopropylvinylboronic acid 3, and 3Å molecular sieves as a water scavenger.[8] Thus, L-3-phenyllactic acid was protected as the acetonide using 2,2-dimethoxypropane and p-toluenesulfonic acid to obtain the (S)-lactone in 96% yield, and subsequently reduced with DIBAL-H to afford the corresponding (S)-lactol 2 in 84% yield.[9] This reaction afforded the anti-diastereomer **4a** exclusively in 53% yield, demonstrating the diastereoselectivity of this transformation. To prepare a probe for the diversification reactions, N-alkylation of **4a** with propargyl bromide was performed to provide the desired compound **5a** in a 72% yield. Next, skeleton-diversifying reactions were performed using different catalysts to provide five 6-APA-based compounds (**6a**-**11a**).

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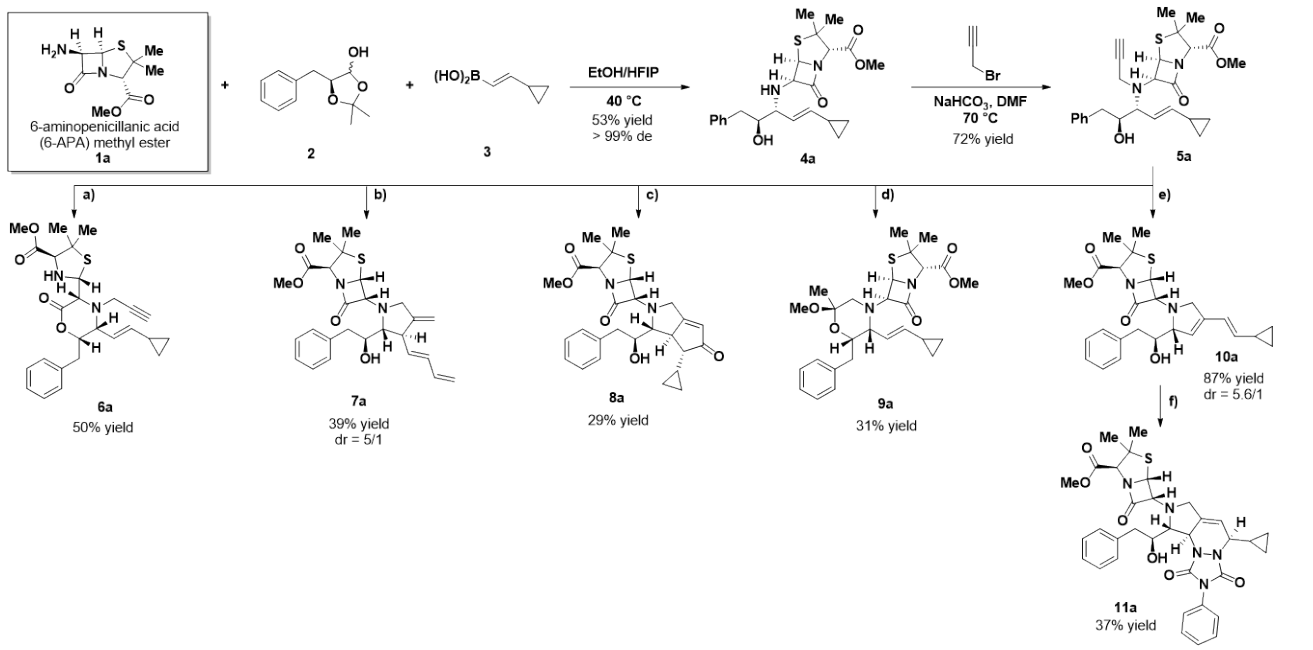

Scheme 1. Outline of the synthesized 6-APA-derived haptens. a) NaH, toluene 0.1 M, rt, 16 h; b) [Pd(PPh3)2(OAc)2] (10 mol%), benzene 0.05 M, 80 °C, 1 h; c) [Co2(CO)8], TMANO, benzene 0.1 M, rt, 4 h; d) NaAuCl4 (10 mol%), methanol 0.05 M, rt, 1 h; e) Grubbs second-generation catalyst (10 mol%), DCM 0.05 M, reflux, 1 h; f) 4-phenyl-1,2,4-triazoline-3,5-dione, DCM 0.1 M, rt, 1 h.

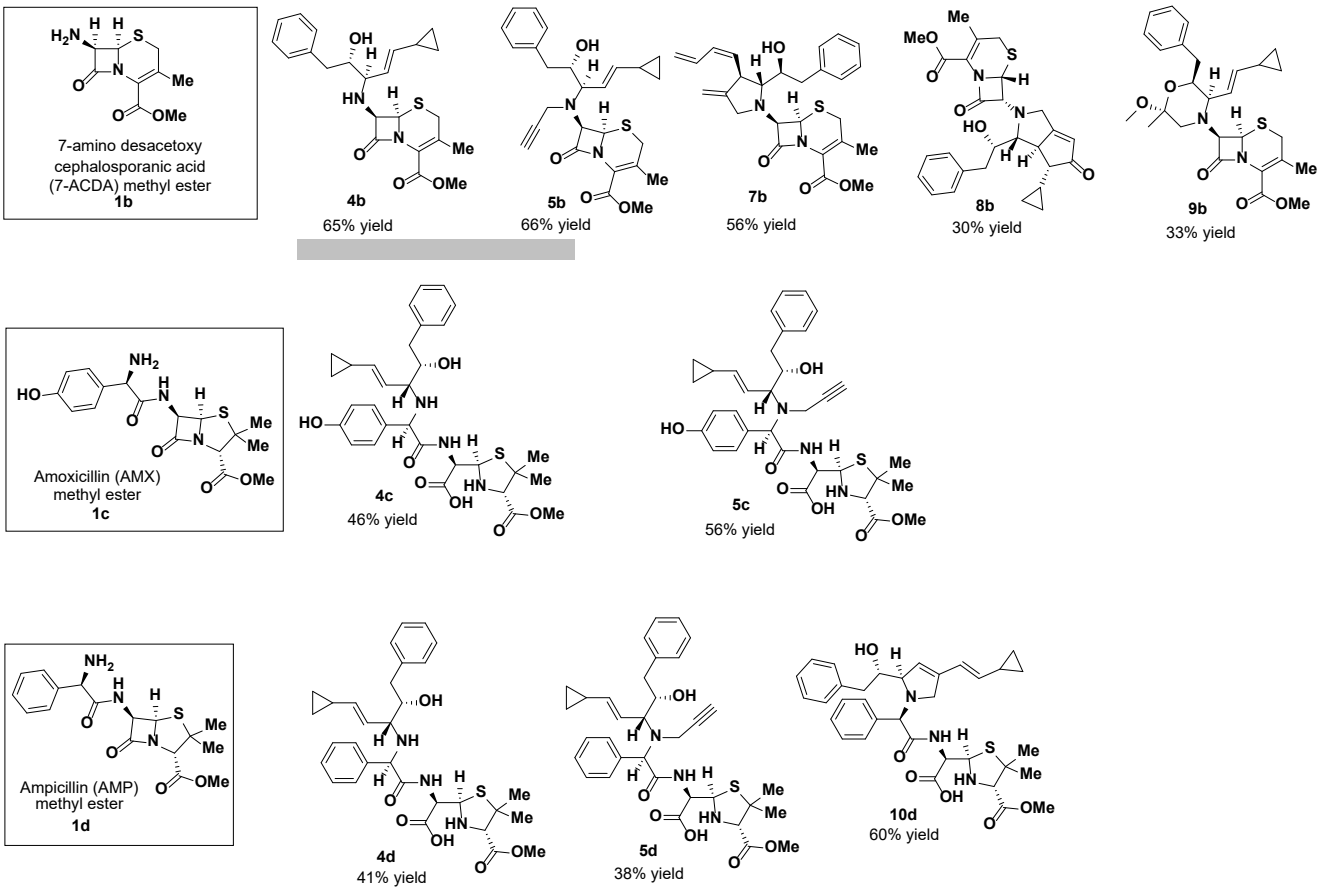

Figure 1. Chemical structures of the synthesised β-lactam-derived haptens.

β-amino alcohols **4b** and **5b** were generated and were subjected to the Pd-catalyzed cycloisomerisation with $[Pd(PPh3)_2(OAc)_2]$, the Pauson–Khand reaction with $[Co_2(CO)_8]$, and the Au-catalyzed alkyne activation and cyclisation to afford structurally diverse compounds **7b**-**9b**, respectively. In the process, we observed the displacement of the double ring in the 3,6-dihydro-2H-1,3-thiazine ring, forming a byproduct that compromised the yield of the reactions.

AMX and AMP, two of the most prescribed β-lactam antibiotics with an additional free amine group on their side chains, were also selected as building blocks and incorporated into the synthetic pathway. We observed that heating at 70 °C for 72 h led to AMX methyl ester (**1c**) and AMP methyl ester (**1d**) decomposition. Mild conditions (room temperature for 72 h) were sufficient to obtain the desired compounds **4c** and **4d** in reasonable yields (ca. 45%), but 2D-NMR studies confirmed that the β-lactam ring opens during the Petasis reaction. After amine propargylation to afford the desired amino alcohols **5c** and **5d** in a reasonable yield (56 and 38%, respectively), cycloadditions were attempted with AMX without success. To assess the feasibility of this pathway, enyne metathesis was chosen as a proof-of-concept in the synthesis of an AMP-based compound, and the desired diene **10d** was obtained in a 60% yield.

The synthesised β-lactam-derived haptens were used to prepare structural antigens following the 'major' configuration with human serum albumin (HSA) and histone (H1) as carrier proteins.[10] A collection of 36 antigens were prepared, 18 for each carrier protein. The antigens were first evaluated using a multiplexed compact disc-based testing tool[10], the heron CD test, with rabbit sera raised for benzylpenicillin (PG) and ceftriaxone (CFT) major antigens. The working principle of the multiplexed CD test is described in detail in the supplementary information. The histogram depicting the ability of all antigens to elicit activity in the immunoassay is shown in Figure S2A and comprises assay data for compounds against sera raised against PG (S1) and CFT (S2). Compounds with assay responses greater than that produced by the negative control were considered 'active.' Fifty percent of the antigens were found to be 'active' with either serum S1 (PG) or S2 (CFT). We next explored whether this activity was serum-specific concerning each β-lactam antibiotic derivative. As depicted in Figure S2B, three of the 36 antigens (**7b**-HSA, **7b**-H1, and **8b**-HSA) were found 'active' across both sera and can be considered generic for detecting IgG. 6-APA-derived antigens were selectively identified by the IgGs of serum S1 with negligible cross-reactivity with S2, with **4a**-, **7a**-, **8a**- and **11a**-derived antigens showing the highest responses independent of the carrier protein used.

A similar pattern was detected for AMX- and AMP-derived antigens. These antigens were also highly selective except for **1d**-H1. This agrees with expectations since 6-APA, AMX, and AMP haptens share a common structure (β-lactam fused to a saturated five-membered ring), considering that serum S1 was raised against the major antigen of benzylpenicillin. On the other hand, **9b**-HSA was the only selective antigen for serum S2, although the specific IgGs stronglyrecognisedd haptens **7b** and **8b**.

Furthermore, we tested a cohort of 31 subjects, of which nine developed an immediate reaction, three suffered from a delayed reaction to β-lactams (Table S2, ESI†), and 19 were control individuals. These patients' serum was tested using two in vitro immunoassays: ImmunoCAP, the gold standard diagnostic test for allergy, and the CD test.[10] None of the 19 control subjects tested positive using either in vitro immunoassay, achieving a specificity of 100%. On the other hand, four allergic patients tested positive for AMX (33%) and three for PG and AMP (25%) using ImmunoCAP. This is consistent with previous reports that show high variability in the sensitivity for ImmunoCAP, especially concerning the β-lactam antibiotics involved (sensitivity values range from 0 to 50 %).[12] We also evaluated the sera of allergic patients using the synthesised haptens with the multiplexed CD test. As shown in Table 1, the antigen prepared with the parent compound 1d was explicitly recognised by IgE of six allergic patients, improving the recognition events compared to that displayed with the major antigens, using either the reference or the CD *in vitro* test. Notably, ten patients tested positive using at least one of the six DOS-derived antigens that incorporate further structural diversity.

Specifically, we obtained positive results for 4 patients for **10a** (33%), 2 for **8a** and **10d** (16%), and 1 for **4b**, **7b**, and **9b** (8 % each). This is perhaps unsurprising given the patients' known allergies to amoxicillin and augmentin (a combination of amoxicillin and clavulanate). Remarkably, specific IgE of 50 percent of the allergic patients explicitly recognised antigens **10a** or **10d**, with these compounds exhibiting more substantial structural differences from their respective antibiotic precursors. Interestingly, both compounds share the same 3-vinyl-2,5-dihydropyrrole moiety arising from enyne metathesis and are the only two compounds tested that contain this group. While anecdotal, this observation suggests that chemically diverse antigens can successfully mimic the epitopes responsible for provoking an allergic episode and demonstrates that even when the chemical structures of the antibiotics have been considerably modified, and the level of specific IgE in serum is very low (< 0.5 IU/mL), patients can be accurately diagnosed as allergic. This reinforces the importance of using the DOS-derived antigens to reach high diagnostic sensitivity. Overall, we have shown that in contrast to other standard antigens, those prepared using DOS-derived haptens were suitable candidate reagents for detecting even low levels of specific IgE in the serum of allergic patients, representing a significant improvement upon the diagnostic sensitivity of in vitro allergy tests, in particular, the reference assay ImmunoCAP. In fact, for the cohort of serum samples analysed, while only four allergic patients (30%) tested positive for amoxicillin with ImmunoCAP, ten tested positive using the DOS-derived antigens (83%) in a multiplex configuration, considering the set of the six selected antigens as a whole what represents a four-fold increase over the reference test. Lastly, 75% of the patients detected as positive by ImmunoCAP also tested positive using the DOS-derived antigens, although the concentration values were significantly different. As seen, DOS-derived antigens underestimate IgE content. However, when

the antigens are combined, the multiplexed CD test identifies a greater number of patients as allergic, becoming them a reasonable alternative to develop accurate *in vitro* diagnostic tests for drug allergy diagnosis. Moreover, the diagnostic sensitivity of 92 % was reached when combining the DOS-derived antigens plus the precursor antigens (**1a-1d**). Besides, all the allergic patients tested positive (100% sensitivity) when the major antigens were also included in the multiplex panel. We designed, synthesised, and performed in vitro immunological evaluation on 18 structurally diverse haptens derived from β-lactam antibiotics. The synthesis of these new haptens and the preparation of the corresponding antigens are critical for accurate β-lactam allergy testing. Furthermore, the elucidation of new antigenic determinants for β-lactam antibiotics might provide new insights into the mechanism involved in allergy and improve the clinical performances of the current in vitro tests. These results suggest that incorporating structural diversity into β-lactam antibiotics can drive molecular IgE recognition—even when the chemical structures of the antibiotics are highly modified and, therefore, the allergic episode, demonstrating the potential of DOS for the preparation of structurally diverse β-lactam-derived haptens. We posit that adopting this strategy could help inform decision-making by allergists, aid β-lactam delabeling initiatives and improve the quality of life of allergic people.

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Conflicts of interest

The authors declare no competing financial interest.

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Table 1. *In vitro* testing of serum samples from allergic patients

	ImmunoCAP[a]				-derived antigens test[a,c]														
	Major				Major				Precursor				DOS						Multiplex
Sample	PG	AMX	AMP		PG	AMX	AMP	CFT	1a	1b	1c	1d	8a	10a	4b	7b	9b	10d	
1	<LD[d]	<LD	<LD		<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	0.33	<LD	<LD	<LD	<LD	+
2	<LD	<LD	<LD		<LD	0.12	<LD	<LD	<LD	<LD	<LD	0.10	<LD	<LD	<LD	<LD	<LD	0.11	+
3	<LD	<LD	<LD		<LD	<LD	<LD	<LD	<LD	<LD	0.23	0.15	<LD	<LD	<LD	<LD	<LD	<LD	+
4	2.16	1.64	1.64		<LD	1.51	<LD	<LD	<LD	<LD	0.50	0.16	<LD	<LD	<LD	<LD	<LD	0.35	+
5	<LD	<LD	<LD		<LD	0.12	<LD	<LD	<LD	<LD	<LD	0.12	<LD	0.19	<LD	<LD	<LD	<LD	+
6	<LD	<LD	<LD		<LD	<LD	<LD	<LD	<LD	<LD	<LD	0.15	<LD	<LD	<LD	<LD	0.37	<LD	+
7	<LD	<LD	<LD		<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	0.20	<LD	<LD	<LD	<LD	+
8	<LD	0.69	<LD		<LD	<LD	<LD	<LD	<LD	<LD	<LD	0.14	0.33	0.31	<LD	<LD	<LD	<LD	+
9	1.83	0.86	1.91		1.25	1.70	1.41	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	+
10	<LD	<LD	<LD		<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	0.14	<LD	<LD	+
11	<LD	<LD	<LD		<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	0.12	<LD	<LD	<LD	<LD	<LD	+
12	0.40	0.54	0.89		0.88	0.69	1.33	0.10	<LD	<LD	<LD	<LD	<LD	<LD	0.11	<LD	<LD	<LD	+

[a]Values correspond to IgE concentration (1.0 IU/mL= 2.4 ng/mL) and are the mean of three replicates. RSD ranged from 13 to 28%; [c]Histone-derived antigens; [d]LD = limit of detection.