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Original preparation of conjugates for antibody production against Amicoumacin-related anti-microbial agents

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ABSTRACT

Amicoumacins are natural products with potent anti-ulcerogenic and anti-bacterial activities, and have been isolated from different *Bacillus* genera. They belong to a family of 3,4-dihydroisocoumarin derivatives bearing hydroxylated amino acid side chains. The 3,4-dihydroisocoumarin moiety of Amicoumacins has been obtained in two steps from 2-methoxybenzoic acid by combining directed and benzylic metalation strategies. The use of *s*-BuLi in both steps gave satisfactory and reproducible yields. For the development of an immunoassay (ELISA) of Amicoumacin-related compounds in biological media, the deprotected 3,4-dihydroisocoumarin moiety has been coupled to the BSA carrier protein via a homobifunctional linker deriving from D-tartaric acid. This approach enabled to introduce the hydroxylated portion of Amicoumacin directly during the preparation of hapten–protein conjugates. The coupling ratio was evaluated by mass spectrometry. The hapten–protein conjugate showing the best coupling ratio was used to generate polyclonal immunosera in rabbits. After immunoserum titration, ELISA conditions were set up and specificity tests were performed on solutions of pure parent compounds, semi-purified Amicoumacin B as well as on culture supernatants of strains known for their Amicoumacin production. This immunoassay is suitable for a rapid and simple screening test for the production of Amicoumacins and its related compounds by bacterial strains.

1. Introduction

Amicoumacin family members represent an unique class of drugs because of their potent anti-ulcerogenic action without any anti-cholinergic and anti-histaminergic effects.[1] Moreover, these compounds possess anti-bacterial, anti-inflammatory, and anti-tumoral activities.[2,3] Amicoumacins belong to a family of 3,4-dihydroisocoumarin derivatives (West block) bearing hydroxylated amino acid side chains (East block) (Fig. 1). Recently, we have demonstrated that the probiotic properties of a *Bacillus subtilis* strain are due to the secretion of Amicoumacin B.[4] The production of Amicoumacin antibiotics by *B. subtilis* is a common characteristic of individual strains that present genetic and physiological homogeneity.[5] Compounds having the same backbone as well as another minor structural analogues have been isolated from other bacterial sources and called AI-77 A, B, C, D, E, and F.[6–9] Amicoumacin A and B (or AI-77 A and B) are the major members of the family. It remains unclear whether the minor structural analogues are natural bacterial metabolites or products of chemical degradation occurring during the isolation of the major compounds. However, it is now well established that Amicoumacin A and B exhibit the highest biological activity.

Amicoumacin B was the principal target of different synthetic approaches and several alternative total syntheses of this compound have been proposed.[10–18] In most of the approaches, the construction of the 3,4-dihydroisocoumarin skeleton was achieved by lithiation at the benzylic position of different *o*-toluic acid derivatives, such as esters[10–13] or oxazolines,[14,15] followed by quenching with N-protected leucinal. In an alternative synthesis, West and East blocks were both assembled from D-ribose as the common chiral source.[16] A convergent and highly stereocontrolled total synthesis of Amicoumacin A, reported by Ghosh, included the preparation of the 3,4-dihydroisocoumarin fragment by a regiospecific Diels–Alder reaction as well as the synthesis of East block by an ester-derived titanium-enolate-mediated *syn*-aldol reaction, a Curtius rearrangement, and the application of Dondoni's

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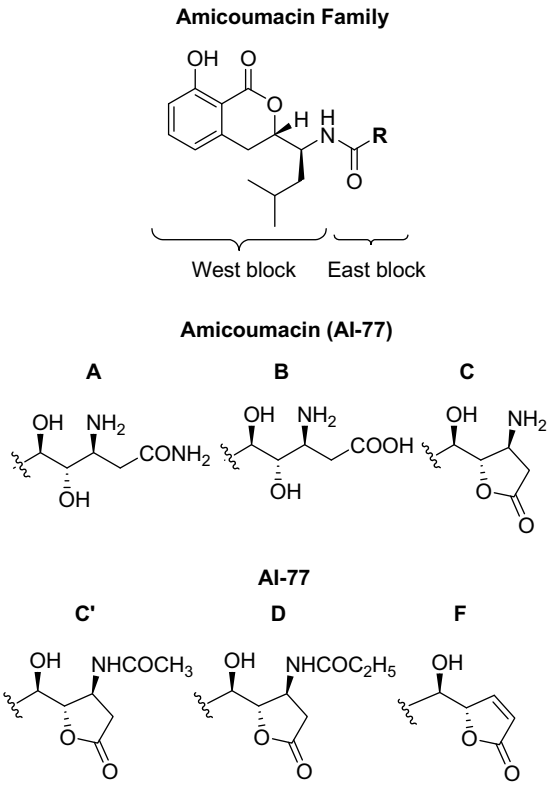


Figure 1. Amicoumacin family compounds.

aldehyde homologation.[17–19] In addition, Superchi et al. described an alternative asymmetric route to the West block based on the regioselective anionic allylation of *N*,*N*′-diethylbenzamides and on the Sharpless asymmetric dihydroxylation.[19,20] Finally, several methodologies have been reported to obtain the East block (three asymmetric carbons).[21–24] However, the total synthesis of Amicoumacin B turned out to be very difficult, expensive, and time consuming, and thus, limited the development of its therapeutic applications. The utilization of bacterial strains producing Amicoumacins may be a good alternative that requires large-scale studies in order to characterize the Amicoumacin production by different bacteria using a simple detection system. However, a reliable and simple system for the quantitative detection of this product is not currently available.

Our work was focused on the development of an easy and rapid detection method for these compounds in biological media. Immunodetection based methods are successfully used for the routine detection of small molecules.[25] ELISA based immunoassays have been previously developed in our Laboratories for the detection of phyto-estrogens in biological media.[26,27]

In this paper, we describe an original preparation of hapten–protein conjugates for the production of specific antibodies against the Amicoumacin family compounds. A short and efficient synthesis of the West block is reported. The deprotected West block was coupled to bovine serum albumin (BSA) via a homobifunctional linker deriving from D-tartaric acid. This approach enabled to introduce the common hydroxylated portion of the East block directly during the preparation of the hapten–protein conjugates. The coupling efficiency was examined by various methods, including UV-spectroscopy, SDS–PAGE and MALDI mass spectrometry. The antibodies raised against the hapten–protein conjugate with the best coupling ratio were evaluated and competitive ELISA in a semi-quantitative format was validated by the detection of semi-purified Amicoumacin B and detection of Amicoumacins in culture media of different *B. subtilis* strains that are known to produce these compounds.[5] This rapid and low cost assay could serve as an indicator of bacterial strains producing Amicoumacins as well as for in vivo studies.

2. Results and discussion

2.1. Immunogen design and synthesis

We were interested in the simultaneous immunodetection of all Amicoumacin analogues in microbiological media. Amicoumacins are low weight molecules, and thus, cannot act as immunogens. The design and synthesis of the corresponding haptens and the hapten–protein conjugates is a crucial step for successful antibody generation. The main structural differences between all Amicoumacins are found on the tail end of the hydroxylated β-amino acid side chain that constitutes the East block. The West block formed by the 3,4-dihydro-8-hydroxyisocoumarin moiety with a leucine analogue at position 3 linked via the amide bond to the part of East Block including the first hydroxy group are common for all major and minor Amicoumacins (Fig. 1).

In order to address the recognition versus the common fragment-derived moiety and to improve the cross-reactivity pattern of the antibodies for the whole Amicoumacin family we designed the immunizing hapten–protein conjugates.

2.1.1. Hapten synthesis

Most of the synthetic approaches toward the West block of Amicoumacins involve the addition of *o*-toluic anions of esters[10–13] or oxazolines[14,15] to N-protected leucinal. These syntheses are time consuming (up to 10 steps including the synthesis of the 2-methoxy-6-methyl-benzoic acid[28]) with moderate yields. Our first attempts concerning the West block synthesis, similar to the original total synthesis reported by Hamada[11] or Ward[13], gave low yields, were often difficult to reproduce, and led to the self-condensation product of benzoic ester, as also mentioned by Kessar.[29]

As underlined by Schlosser and Geneste, '*The best protective group is the one which can be omitted*'.[30] Directed *ortho*-metalation of unprotected benzoic acids has become an important and useful method for the preparation of different polyfunctional aromatic systems.[31–35] On the other hand, reactions of metalated toluic acids with different electrophiles have been less explored.[36–39]

We decided to explore the combined directed and benzylic metalation strategy on unprotected benzoic acids for the synthesis of the West block of Amicoumacins. The 3,4-dihydroisocoumarin moiety was obtained in only two steps from commercially available 2-methoxybenzoic acid **1** by a regioselective *ortho*-lithiation reaction,[31,32] followed by the generation of the benzylic carbanion of **2**, and then by the addition to *N*-Boc-protected leucinal **3** (prepared as described by Dondoni et al.[40]) with a spontaneous lactone formation during a mild acid hydrolysis (Fig. 2).

In the benzylic metalation step, the lithio species was generated by treatment of **2** with different lithium bases (Table 1 in Supplementary data). The use of *s*-BuLi gave satisfactory and reproducible yields (39%, **4** + **5**) as well as reasonable diastereoselectivity comparable with previous syntheses. The use of 3 equiv of *s*-BuLi seems to be crucial for the good deprotonation of **2**. The yield of the reaction was not improved by addition of TMEDA and the utilization of sparteine[41] did not increase the diastereoselectivity. It is worthy to note that the use of *o*-toluic acid instead of **2** under the same conditions as entry **8** (Table 1 in Supplementary data) led to dihydroisocoumarin formation only in a 24% yield. This observation is in good agreement with the study of possible competition between *ortho*- and lateral-lithiation of *o*-toluic acid derivatives.[42]

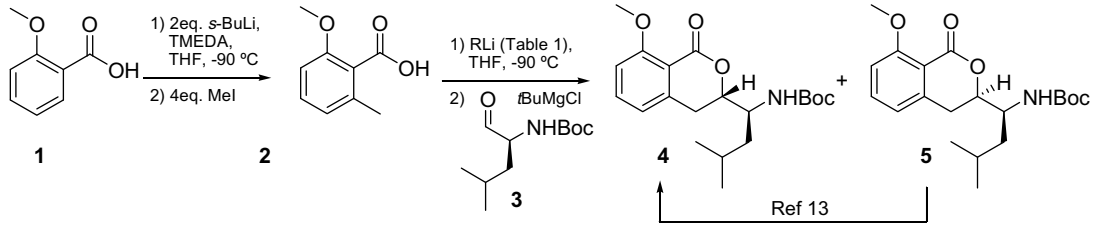


Figure 2. Synthesis of the protected West block **4** of Amicoumacins.

The preliminary treatment of **3** with *t*-BuMgCl was crucial to obtain a reproducibility of the benzylic anion addition. The N-deprotonation of **3** is presumed to stabilize the chelation model according to Cram's rule and explains the reasonable diastereoselectivity. The conversion of **3** to the *N*-lithio derivative prior to the addition of the benzylic anion, as described for *N*-benzylsulfonyl-leucinal,[11] did not lead to the expected product.

The stereochemistry of the major product **4** was confirmed by comparison of its physical characteristics with literature data[13] ($[\alpha]_D$ −118° (*c* 1, MeOH); lit. $[\alpha]_D$ −122.8° (*c* 2.02, $CHCl_3$); ^1H RMN spectrum and IR spectrum). The compound **5** with the undesirable configuration was converted into **4** as previously reported.[13] The deprotection of the Boc function and demethylation of **4** was achieved by reaction with boron tribromide in dichloromethane at −78 °C (Fig. 3).[10] The basic treatment of **6**·HBr gives the free amine **6**, which easily isomerizes to the seven-membered lactam **7**, as previously noted in the degradation study of natural Amicoumacins.[9] The lactam **7** can be transformed to **6**·HCl only at high temperature.

In summary, we have developed a new synthetic pathway to the West block of Amicoumacins with major advantages compared to previous syntheses[11,13,15] (see Table 2 in Supplementary data).

2.1.2. Conjugation to the carrier protein

For the production of specific antibodies against the Amicoumacin family, the deprotected 3,4-dihydroisocoumarin moiety **6**·HBr was coupled to the bovine serum albumin (BSA) carrier protein via a homobifunctional linker deriving from D-tartaric acid **8** (Fig. 4). By this method, part of the hydroxylated portion of the Amicoumacin East block is introduced directly during the preparation of the hapten–protein conjugates. This approach should enable to mimic the common portion of all compounds of the Amicoumacin family and to increase the sensitivity of the immunoresponse with no discrimination of particular members of the family.

Compound **8** was freshly prepared from D-tartaric acid with a good yield (80%) using the typical procedure for *N*-succinimidyl ester synthesis described by Anderson et al.[43] The synthesis of the West block-BSA conjugates was performed at different molar ratios of **6**/BSA 50:1 (**9a**) and 100:1 (**9b**) to determine the optimal ratio for further routine use in immunogen and coating antigen preparation. The conjugation reaction is crucial for the success of an immunization, and the use of large excess of hapten ensures the high substitution of the lysine side chains of the carrier protein. On the other hand, the hapten overload is not reasonable (unreacted West block is lost during the conjugate purification) because of the complexity of its synthesis that requires the utilization of organolithium reagents.

2.1.3. Analysis of the BSA conjugates

Considering the three-dimensional structure of BSA, only 26 ε-NH_2 groups of surface lysines (out of 59 lysine residues) of BSA are theoretically available for coupling with haptens.[44] A high ratio of hapten per molecule of BSA ensures the efficiency of the coupling reaction. Therefore, it is very important to verify the efficiency of coupling before its administration to rabbits for antibody generation because the use of homobifunctional linkers increases the risk of undesired cross-linking between the same molecules. The conjugates were analyzed by three independent methods: UV-spectroscopy, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and matrix-assisted laser desorption–ionization mass spectrometry (MALDI-MS) (Table 1).

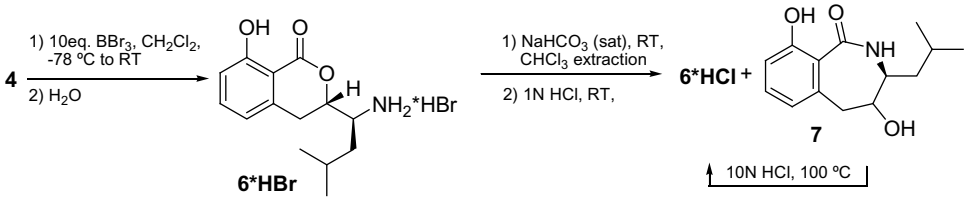


Figure 3. Removal of the protecting groups of the West block.

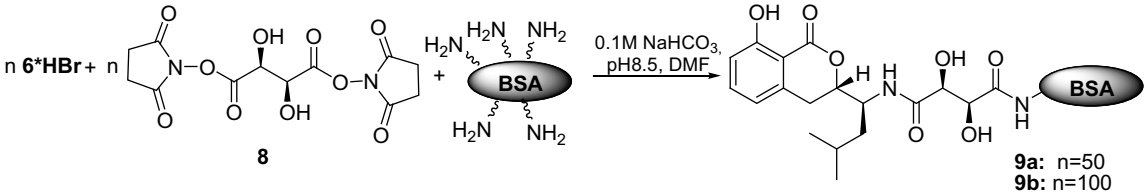


Figure 4. Preparation of the Amicoumacin–protein conjugates.

Table 1
Comparison of hapten incorporation on BSA conjugates determined by SDS–PAGE electrophoresis and MALDI-MS

Conjugate	Hapten:BSA[a]	Calculated hapten number per molecule of BSA ($MW_{conjugate} - MW_{BSA}$)/$MW_{hapten}$						
		SDS–PAGE[b]		MALDI-MS				
		Exp. 1	Exp. 2	Matrix 1[c]		Matrix 2[d]		Average
				Ion 2^+	Ion 1^+	Ion 2^+	Ion 1^+	
9a	50: 1	8.3	4.5	9.3	9.25	10.7	10.6	10.0
9b	100: 1	6.4	7.5	10.5	10.3	11.8	11.4	11.0

[a] Ratio used in conjugate preparation.
[b] Molecular weights were calculated from peak centroids generated from SDS–PAGE bands with BioRad GS-800 Quantity One program from two independent experiences.
[c] α-Cyano-4-hydroxy-cinnamic acid.
[d] Sinapinic acid.

The UV spectra display the differences between the carrier protein and the conjugates in the regions of the maximum absorbance of the hapten, and provide a qualitative characterization of the coupling efficiency (see Supplementary data). Conjugates were also analyzed by SDS–PAGE. For the conjugates, smeared bands along with weak bands for polymerized BSA were observed (see Supplementary data). The molecular weight changes were determined for each conjugate and the calculated hapten number per molecule of BSA is presented in Table 1. The small changes detected in the molecular weight can be explained by the poor accuracy of SDS–PAGE (5%), moreover, the hapten density determined by this method varies a lot for the same sample preparation loaded in two different gels. SDS–PAGE does not reflect the real degree of hapten incorporation because it is insensitive to small changes in molecular weight. The main advantage of this method is the detection of BSA self cross-linking as a side product during the conjugation reaction.

MALDI-MS gave the best determination of hapten conjugation. The average molecular weight of each conjugate was calculated from both singly $(M+H)^+$ and doubly $(M+2H)^{2+}$ charged peaks using two different matrices (Table 1). This method enabled to determine the average number of bound molecules with high reproducibility and accuracy.

Noticeably, around 10–11 hapten molecules were bound to BSA, and that this number did not change when the hapten to protein coupling ratio used was increased. This observation was confirmed by both SDS–PAGE and MALDI-MS. Thus, only part of the 26 theoretically available surface lysines of BSA is accessible for coupling with haptens under our conditions and the utilization of 50 equiv of hapten is sufficient enough to saturate available sites on protein surface.

2.2. ELISA development

2.2.1. Antibody titer determination

The hapten–protein conjugate **9a** was used to generate polyclonal antibodies in two germ-free, pathogen-free rabbits. Comparison of the binding of the two polyclonal immunosera with the immobilized antigen coated onto the plates demonstrated that immunosera 4676 was twice more concentrated than 4677 (titer × 2.16). Therefore, the former was retained for further tests, and Figure 5 shows the results of the direct revelation test. It must be noted that 1/1000 dilutions of each immunoserum were out of range indicating a good response to the injection of the hapten–protein conjugate. The coating with hapten–Thyr conjugates at 0.25 μg/mL and the 1/5000 dilution of antisera (final well dilution

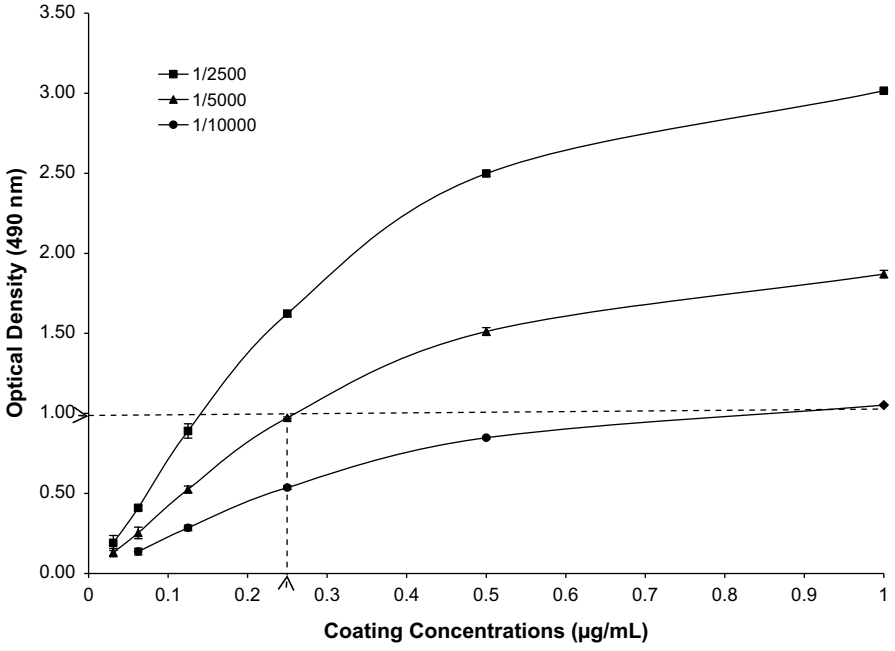


Figure 5. Cross-test for titer determination applied to serum 4676. This is a direct measurement of the maximal binding for each couple of antisera and immobilized antigen concentrations. For each data $n = 4$ and they are presented ±SD.

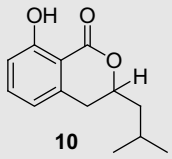
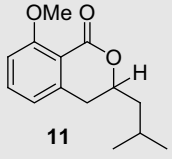
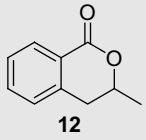
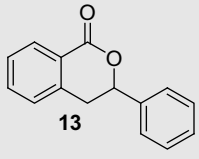
1/10,000) were used as standard conditions for preliminary competitive ELISA tests.

2.2.2. Cross-reactivity

Specificity of the immunoassay was assessed by measuring the interference of some structurally related substances or microbiological preparations from *B. subtilis* strains with the immobilized hapten (hapten–Thyr) for the reaction with the polyclonal antibody (Table 2). It is a competitive assay and values are expressed as percentages of total binding (Bo) obtained when the antibody is only challenged with hapten immobilized on the wells and without any competitive solution. Bi is the OD obtained when the antibody is challenged with both the immobilized hapten and the unknown solution. Note that the non-specific binding is taken into account in Blank wells where the antibody was present without the immobilized antigen. Therefore data are expressed as a percentage of maximum binding, that is (Bi − Blank)/(Bo − Blank). These transformations of the data amplify the differences observed between the different compounds. When the unknown compound is not recognized by the antibody there is no interference with the immobilized antigen and the binding is close to 100%. When there is affinity of the unknown compound for the antibody, the latter reacts preferably with the free compound in solution rather than with the immobilized hapten and the percentage of binding is reduced. When a large set of dilution of the antigen is challenged with the immobilized hapten for the antibody, a classical sigmoid curve is obtained (data not shown). Classically, biochemical significance is obtained for percentages of binding lying in the linear portion of the sigmoid (i.e., between 30% and 75% of binding). Over and below these values since the values, are no longer in the linear portion of the curve, the binding is no more proportional to the affinity of the antibody for the unknown compound. The percentages of binding obtained in these conditions allow classifying the affinity of the unknown samples for the polyclonal antibody generated. To determine the specificity of the antibody, several parent compounds **10**–**13** (entries 10–13, Table 2) were specially synthesized by direct benzylic metalation of corresponding unprotected benzoic acids (see Supplementary data). Semi-purified Amicoumacin B and culture supernatants of Amicoumacin producing strains were used as a positive control. Compounds **6**·HCl and **6**·HBr, representing a major part of the Amicoumacin West block, induce a significant displacement of the binding of the antibody to the immobilized antigen. For **6**·HCl the displacement reaches 35%. The 3-isobutyl-8-hydroxy-3,4-dihydroisocoumarin **10**, which is a non-natural compound with a high degree of homology to the West block, displaces the antibody in the same manner as **6**·HBr. The fully protected West block precursor **4** (entry 9, Table 2) was not recognized. No significant cross-reactivity was observed with other synthetic 3,4-dihydroisocoumarin derivatives tested (compounds **11**, **12**, and **13**). These data indicate a good specificity of antibodies that are able to selectively recognize the 8-hydroxy-3,4-dihydroisocoumarin moiety. The compounds with another substitution in position 8 were not discriminated. The presence of an alkyl group in position 3 is also important for good recognition.

In summary, the cross-reactivity observed with different chemical analogues of Amicoumacins confirmed that our immunogen provided a favorable antigen presentation on the protein surface and the specificity of the antibodies raised against the conjugate **9a** is sufficient for qualitative determination of Amicoumacin family.

Table 2
Validation of the ELISA test: reactivity of antiserum with structurally related chemicals and microbiological samples

Entry	Analyte	% of maximum binding to coated antigen: (Bi − Blank)/(Bo − Blank) * 100[a]
1	Semi-purified Amicoumacin B	3.2
2[b]	*B. subtilis* BS3	7.8
3[b]	*B. subtilis* BS3 (diluted 1024)	41
4[b]	*B. subtilis* M1-2	20
5[b]	*B. subtilis* 168	77
6[b]	*B. subtilis* BM15	83
7	**6**·HCl	65
8	**6**·HBr	75
9	**4**	97
10[c]	 **10**	73
11[c]	 **11**	95
12[c]	 **12**	91
13[c]	 **13**	100
14	Culture media	87

[a] Bi, Bo, and Blank are the OD measured in the well at 490 nm. Bo is maximum binding to the immobilized hapten-Thyr without any other compound added, Bi is the OD obtained when the unknown compound is added, Blank is the non-specific binding. When the unknown compound is not recognized by the antibody there is no interference with the immobilized antigen and the binding is close to 100%.

[b] Culture supernatant of *Bacillus* strain.

[c] Chemical synthesis and structural characteristics are given in Supplementary data.

2.2.3. ELISA validation for the detection of Amicoumacins

The ELISA test was validated by using a quantitative analysis of Amicoumacins in culture supernatants of *Bacillus* strains that were previously identified as positive or negative producers of these compounds.[5] Culture supernatants from negative Amicoumacins production strains *B. subtilis* 168 and BM15, as well as bacterial culture media did not significantly displace the antibody from its binding to the immobilized antigen (Table 2). In contrast, positive results in the ELISA test were obtained with semi-purified Amicoumacin B, and strains BS3 and M1-2 known for their production of Amicoumacins.

Pinchuk et al.[5] demonstrated that anti-microbial activity of BS3 strain against *S. aureus* is related to the Amicoumacin B production. Titer of BS3 supernatant against *S. aureus* was obtained using a classical half dilution method as described by Pinchuk et al.[4,5] Using this method, we detected 64 Arbitrary Units (AU) in the BS3 supernatant that correspond to a supernatant diluted 64 times (data not shown). In contrast, the ELISA developed enabled to detect Amicoumacin in BS3 culture supernatant diluted 1024 times (1024 AU). Thus, the developed ELISA test is about 20 times more sensitive than the Amicoumacin detection previously described.[5]

3. Conclusions

The goal of this work was the development of an ELISA test for the detection of Amicoumacins for rapid and easy screening of bacterial producers and for future in vivo studies. The West block was selected as principal common epitope for raising antibodies. A new short and efficient synthesis of the Amicoumacin West block and an original strategy for the preparation of Amicoumacin-protein conjugates are described. As expected, the use of a D-tartaric acid derivative as a coupling reagent enabled to mimic the common portion of all of the compounds of the Amicoumacin family and to increase the sensitivity of the immunoresponse without discriminating any particular member of the family. MALDI-MS was a very useful method for precise measurement of the hapten/BSA ratio.

Our ELISA test for rapid and low cost detection of Amicoumacin in biological samples uses a four-step procedure, and compared to the classic method[5] produces results in 3 h with a 20 times higher sensitivity. The assay has applications as a specific screen for Amicoumacin producers among different bacterial strains, as well as to study in vivo metabolism and bioavailability of these compounds.

4. Experimental

4.1. Chemicals and instruments

2-Methoxybenzoic acid (99%), TMEDA (99%), methyl iodide (99%), *tert*-butylmagnesium chloride (2.0 M in diethyl ether), and boron tribromide (99%) were purchased from Sigma Aldrich Chemical Co. (Saint Quentin Fallavier, France), while 4-hydroxyphenylacetic acid (98%) was purchased from Acros Organics France (Noisy, France). All other chemical reagents used were from Lancaster Synthesis Ltd (Bishheim, France).

Melting points were determined with a Stuart Scientific melting point apparatus SMP3 and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded with a Bruker AC-300 FT (^{1}H: 300 MHz, ^{13}C: 75 MHz) using TMS as an internal standard. The chemical shifts (δ) and coupling constants (J) are, respectively, expressed in ppm and Hz. IR spectra were recorded with a Perkin-Elmer paragon 1000 FT-IR spectrophotometer. Optical rotations were measured on Bellingham Stanley Polarimeter ADP220 at ambient temperature. Mass spectra (both high and low resolution) were acquired on a QStar Elite mass spectrometer (Applied Biosystems). The instrument was equipped with an electrospray ionization (ESI) source and spectra were recorded in the positive mode. The electrospray needle was maintained at 4500 V and operated at room temperature. Samples were introduced by injection through a 10 μL sample loop into a 200 μL min^{-1} flow of methanol from the LC pump. Thin-layer chromatography (TLC) was performed using SDS TLC plates, 0.25 mm, particle size 15 μm, pore size 60 Å. Merck silica gel 60 (70–230 mesh) and (0.063–0.200 mm) were used for flash chromatography. The spots were visualized with UV lamp or revealed with ninhydrin. Diethyl ether and THF were distilled over sodium wire and benzophenone under argon atmosphere immediately before use. Dichloromethane was distilled over CaH_2. *n*-BuLi (2.5 M solution in hexane) and *s*-BuLi (1.3 M solution in cyclohexane/hexane) were purchased from Acros Chemical Co., Inc. and used after titration with diphenylacetic acid. All moisture-sensitive reactions were performed under argon atmosphere in oven-dried or flame-dried glassware.

4.2. 2-Methoxy-6-methyl-benzoic acid (2)

To a 500 mL round-bottomed flask equipped with a condenser and a rubber septum under argon, freshly redistilled TMEDA (12.8 g, 16.6 ml, 0.11 mol, bp 120.5–121.0 °C) and 100 mL of dry THF were added. The mixture was stirred magnetically and cooled to −90 °C. After consecutive addition of *s*-BuLi (0.11 mol, 1.3 M in cyclohexane/hexane, 85 mL) and 2-methoxybenzoic acid (7.6 g, 0.05 mol) in 30 ml of dry THF at −90 °C, the orange solution was stirred for an additional hour. Methyl iodide (28.4 g, 12.45 ml, 0.20 mol) was added dropwise for 30 min. After stirring for 2 h, the mixture was then treated with water, washed with diethyl ether, and shaken, and the aqueous layer was acidified with 1 N HCl down to pH 2. The mixture was diluted with diethyl ether, and the organic layer was separated and dried with $MgSO_4$. Filtration, concentration in vacuo, and recrystallization (diethyl ether/pentane) gave **2** (4.15 g, 25 mmol, 50%). mp 138–140 °C (lit. 139–140 °C[45]). ^{1}H NMR ($CDCl_3$) δ_H 11.58 (1H, O*H*); 7.30 (t; 1H; J = 8.1, Ar*H*); 6.88 (d; 1H, J = 7.6, Ar*H*); 6.83 (d; 1H, J = 8.2, Ar*H*); 3.91 (s, 3H, –O–CH_3); 2.49 (s, 3H, Ar-C***H*₃**). NMR-^{13}C ($CDCl_3$) δ_C 170.3, 155.8, 137.4, 130.2, 122.3, 107.7, 107.8, 55.8, 19.2. IR ν (cm^{-1}) 3007, 1697, 1595, 1470, 1293, 1269, 1092, 1073, 915, 788. Spectral characteristics were identical to those previously published.[31]

4.3. (3*S*)-3-[(1′S)-*tert*-butyloxycarbonylamino-3′-methylbutyl]-8-methoxy-3,4-dihydroisocoumarin (4)

s-Butyllithium (l4.4 mmol, 1.3 M in cyclohexane/hexane, 11.1 ml) was added dropwise to a solution of **2** (0.77 g, 4.64 mmol) in 30 ml of dry THF at −90 °C. A bright red color appeared and the solution was stirred for 45 min. In another flask, *tert*-butylmagnesium chloride (14.5 mmol, 2 M in diethyl ether, 7.3 ml) was added to (*S*)-2-*tert*-butyloxycarbonylamino-4-methyl-1-pentanal **3** (3.10 g, 14.4 mmol) in 10 ml of dry THF at −78 °C and stirred for 5 min before addition via cannula to the lithiated compound **2**. The bright red coloration disappeared progressively. The mixture was stirred for a further 2 h at −90 °C and allowed to warm to room temperature. Saturated aqueous ammonium chloride solution (100 ml) was added and pH was adjusted to pH 2–3 with 1 M hydrochloric acid. The vigorous stirring was maintained for 1 h 30 min at room temperature, then the solution was extracted with ethyl acetate (3× 50 ml). The combined organic extracts were washed with water (50 ml) and brine (50 ml), dried ($MgSO_4$), and concentrated under reduced pressure. Flash chromatography using pentane–diethyl ether (4:1) as an eluent gave the desired compound **4** as a pale yellow oil (0.51 g, 1.40 mmol, 30%, R_f = 0.08). ^{1}H NMR ($CDCl_3$) δ_H 7.42 (t, 1H, J = 8.3, Ar*H*); 6.88 (d, 1H, J = 8.6; Ar*H*); 6.79 (d, 1H, J = 7.5, Ar*H*); 4.85 (d, 1H, J_{NH-CH} = 9.8, –NH–CH–); 4.34 (d, 1H, J = 12.4, –O–CH–); 3.91 (s, 3H, Ar-OCH_3); 3.65–3.60 (m, 1H, –NH–CH–); 3.02 (dd, 1H, J = 16, 11.4, H_{4b}); 2.75 (dd, 1H, J = 16, 2, H_{4a}); 1.73–1.60 (m, 1H, $H_{2'b}, H_{3'}$); 1.43–1.50 (m, 1H, $H_{2a'}$); 1.41 (s, 9H, *t*-Bu), 0.91 (d, 6H, J = 8.0, CH-(CH_3)$_2$). ^{13}C NMR ($CDCl_3$)δ_C 170.1, 161.3, 156.6, 142.2, 134.6, 118.5, 113.4, 107.7, 79.7, 79.5, 55.9, 50.5, 41.0, 31.7, 28.3, 24.7, 22.9, 22.0. IR ν, cm^{-1} 3438, 3325, 2959, 2932, 2870, 1707, 1599, 1507, 1477, 1367, 1277, 1238, 1169, 910, 733. MS (ESI$^+$) m/z 749 [2M+Na]$^+$, 386 [M+Na]$^+$; HR-MS (ESI$^+$) m/z calcd for $C_{20}H_{29}NO_5$, 386.1937 [M+Na]$^+$; found, 386.1941. $[\alpha]_D$ −118° (*c* 1, MeOH).

The followed fraction gave the minor diastereoisomer **5** (3*R*)-3-[(1′S)-*tert*-butyloxycarbonylamino-3′-methylbutyl]-8-methoxy-3,4-dihydroisocoumarin. This product was isolated as a pale yellow oil (0.17 g, 0.464 mmol, 9%, R_f = 0.05). ^{1}H NMR ($CDCl_3$) δ_H 7.43 (t, 1H, J = 8.3, Ar*H*); 6.89 (d, 1H, J = 8.6, Ar*H*); 6.81 (d, 1H, J = 7.6, Ar*H*); 4.89 (br d, 1H, J_{NH-CH} = 9.8, –NH–CH–); 4.36 (d, 1H, J = 9.8, –O–CH–); 3.91 (s, 3H, Ar-OCH_3), 3.72–3.69 (m, 1H, –NH–CH–); 2.96 (br d, 1H, J = 12.4, H_{4b}); 2.77 (dd, 1H, J = 16, 2.5, H_{4a}); 1.47–1.80 (m; 3H; $H_{2'a}, H_{2'b}, H_{3'}$); 1.42 (s, 9H, *t*-Bu), 0.93 (d, 3H, J = 7.2; –CH–(CH_3)$_2$); 0.91 (d, 3H, J = 7.2; –CH–(CH_3)$_2$). ^{13}C NMR($CDCl_3$) δ_C 169.3, 160.1, 154.6, 141.3, 137.1, 122.1, 118.4, 112.7, 79.4, 78.8, 55.1, 49.9, 40.1, 30.7, 27.3, 23.7, 22.7, 20.3. IR ν/cm^{-1} 3326, 2959,

1707, 1596, 1508, 1477, 1238, 1169, 911, 730. MS (ESI$^+$) *m*/*z* 386 [M+Na]$^+$, 364 [M+H]$^+$.

4.4. (3*S*)-3-[(1′*S*)-*t*-butyloxycarbonylamino-3′-methylbutyl]-8-hydroxy-3,4-dihydroisocoumarin hydrobromide (6·HBr)

Boron tribromide (400 mg, 150 μl, 1.6 mmol) was added dropwise to a solution of **4** (140 mg, 0.4 mmol) in 30 ml of dichloromethane at −78 °C over 5 min. The mixture was stirred for 30 min at −78 °C. After addition of distilled water (10 ml), the mixture was allowed to warm to room temperature and extracted with water (3× 10 ml). The combined aqueous extracts were lyophilized to give a light brown powder (110 mg, 85%). After recrystalization from ethanol light brown crystals were obtained. ^{1}H NMR (MeOH-d_4) δ_H 7.53 (t, 1H, *J* = 8.4, Ar*H*); 6.92 (d, 1H, *J* = 8.6, Ar*H*); 6.89 (d, 1H, *J* = 8.2, Ar*H*); 4.76 (m, 1H, –O–CH–); 3.60–3.57 (m, 1H, –NH–CH–); 3.20–3.05 (m, 2H, *H*$_4$); 1.90–1.75 (m, 1H, $H_{3'}$); 1.71–1.60 (m, 2H, $H_{2'a}$, $H_{2'b}$); 1.04 (d, 3H, *J* = 6.3, –CH–$(CH_3)_2$); 1.02 (d, 3H, *J* = 6.3, –CH–$(CH_3)_2$). ^{13}C NMR (MeOH-d_4) δ_C 169.8, 159.9, 139.6, 136.3, 120.6, 116.1, 108.4, 83.5, 52.0, 42.6, 30.0, 24.5, 23.6, 21.5. IR *ν*, cm^{-1} 3364, 2956, 1685, 1462, 1259, 1025, 799. HR-MS (ESI$^+$) *m*/*z* calcd for $C_{14}H_{19}NO_3$, 250.1437 [M+H]$^+$; found, 250.1431.

4.5. 6·HCl and (3S,4S)-4,9-Dihydroxy-3-isobutyl-2,3,4,5-tetrahydro-benzo[*c*]azepin-1-one (7)

The lyophilized crude **6**·HBr (100 mg, 0.3 mmol) was treated with saturated aqueous sodium hydrocarbonate (15 ml). The solution was extracted with dichloromethane (3× 25 ml), then the combined organic layers were dried over $MgSO_4$. After filtration, the organic layer was concentrated under reduced pressure to give a 3/1 mixture of **6** and **7** as a brown oil, which was dissolved in 10 ml of MeOH and treated with 5 ml of 1 N HCl. After concentration under reduced pressure, chromatography of the residue using hexane–ethyl acetate (3:7) as an eluent followed by elution with methanol gave **6**·HCl (65 mg, 65%) (fraction eluted with MeOH) as a white solid and the compound **7** as a colorless oil (22 mg, 22%). The product **7** was quantitatively transformed into **6**·HCl by heating with concd HCl at 100 °C for 2 h.

6·HCl: physical and NMR characteristics were identical to those previously published.[11,13]

4.5.1. (3*S*,4*S*)-4,9-dihydroxy-3-isobutyl-2,3,4,5-tetrahydro-benzo[*c*]azepin-1-one (7)

^{1}H NMR (MeOH-d_4) δ_H 7.27 (t, 1H, *J* = 8.3, ArH); 6.83 (d, 1H, *J* = 8.3, ArH); 6.78 (d, 1H, *J* = 8.3, ArH); 4.03 (m, 1H, –C(4)HOH–); 3.21–2.95 (m, 2H, –NH–C(3)H–; H_{5a}); 2.72 (dd, 1H, *J* = 13, 9.8, H_{5b}); 1.81–1.55 (m, 2H, $H_{2'a}$, $H_{3'}$); 1.47–1.28 (m, 1H, $H_{2'b}$) 0.91 (d, 3H, *J* = 6.8, –CH–$(CH_3)_2$); 0.81 (d, 3H, *J* = 6.4, –CH–$(CH_3)_2$). ^{13}C NMR (MeOH-d_4) δ_C 174.2, 159.9, 137.6, 132.4, 120.1, 116.2, 115.1, 75.3, 54.5, 40.4, 37.8, 24.3, 21.9, 20.8. IR *ν*, cm^{-1} 3421, 2956, 2526, 1635, 1457, 1212, 1115, 971. MS (ESI$^+$) *m*/*z* 250 [M+H]$^+$.

4.6. (2*S*,3*R*)-2,3-Dihydroxy-succinic acid bis-(2,5-dioxo-pyrrolidin-1-yl) ester (8)

Dry D-(−)-tartaric acid (1.5 g, 10 mmol) and *N*-hydroxy-succinimide (2.5 g, 22 mmol) were dissolved in 30 ml of dry THF and cooled to 0 °C. A solution of dicyclocarbodiimide (4.5 g, 22 mmol) in 20 ml of dry THF was added dropwise. The mixture was allowed to warm to room temperature then stirred for 16 h. The precipitated dicyclohexylurea was filtered off, the filtrate was evaporated under reduced pressure and a residue was triturated with dry EtOH to afford **8** as a white solid (2.4 g, 70%, mp 198 °C). The product was very hydroscopic, was kept in desiccator and used rapidly. ^{1}H NMR (DMSO-d_6) δ_H 6.73 (d, 2H, *J* = 7.5, O*H*); 4.89 (d, 2H, *J* = 7.5, *CH*OH); 2.83 (s, 8H, $-CH_2-CH_2-$). ^{13}C NMR (DMSO-d_6) δ_C 170.1, 167.0, 61.9, 26.3. Spectral characteristics were identical to those previously published.[46]

4.7. Preparation of hapten–BSA conjugates (9a and 9b)

Conjugates were prepared at two different ratios of hapten to BSA (50:1 for **9a** and 100:1 for **9b**). BSA (33.5 mg, 0.5 μmol) was dissolved in 5 ml of 0.1 M $NaHCO_3$ (pH 8.5) and cooled to +4 °C. **6**·HBr (8.3 mg, 25 μmol for **9a** and 16.5 mg, 50 μmol for **9b**) in DMF (0.5 ml) was added. Freshly prepared **8** (8.6 mg, 25 μmol for **9a** and 17.2 mg, 50 μmol for **9b**) was dissolved in dry DMF (0.2 ml) and added to the mixture. After stirring at +4 °C for 4 h, the mixture was dialyzed (molecular weight cut off 6000–8000 Da) against distilled water (2× 1000 ml) at +4 °C for 48 h and lyophilized to afford a white powder (35 mg for **9a** and 36 mg for **9b**). The products were stored at −20 °C.

4.8. Coating antigens

The swine thyroglobulin (Thyr) was used for the preparation of the coating antigen. The compound **6**·HCl was attached to Thyr by the same method as described above for **9a** and **9b** except for (1) the stoichiometric ratio hapten/Thyr was 200:1 and (2) the protein solution was prepared in borate–boric buffer (0.2 M, pH 8.7).

4.9. Characterization of conjugates

The conjugates **9a** and **9b** were analyzed by UV-spectroscopy, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), and matrix-assisted laser desorption–ionization mass spectrometry (MALDI-MS). *UV-spectroscopy:* the UV spectra were run with a UV/vis spectrophotometer (Hitachi U-3300). The samples were prepared at a concentration of 1 mg/mL in distilled water. *SDS–PAGE:* conjugates were dissolved in the SDS–PAGE sample buffer (10 mM Tris/HCl, 1 mM EDTA, 2.5% SDS, and 5.0% β-mercaptoethanol, pH 8.0) to 1 mg/mL and heated at 100 °C for 5 min. Gels were loaded with 2–3 μl of the sample and run under denaturing conditions then were stained with Coomassie Blue according to the manufacturers' recommendations. The resulting gels were evaluated first by scanning and second by analyzing the gel image, with BioRad GS-800 Quantity One program. From the gel image a graphical presentation of the gel bands observed was produced and the molecular weights from peak centroids were calculated. *MALDI-MS:* mass spectra were recorded in the linear mode with a Bruker Reflex III mass spectrometer equipped with a UV laser (337 nm). Samples (10 μM) were mixed (1/1 v/v) with matrix solutions (sinapinic acid or α-cyano-4-hydroxy-cinnamic acid, 10 mM in water/acetonitrile) and 1 μl of the mixture was applied on a stainless steel target and left to dry at room temperature. Spectra were acquired in the external calibration mode using BSA as a reference.

4.10. Antibody production

The polyclonal antibodies were raised in two different rabbits against the BSA–hapten **9a** coupled in a borate–boric buffer with a coupling ratio of 1/100. Immunizations were achieved in regular house holding conditions and on sanitary controlled New Zealand rabbits from Millegen (France). Rabbits were first ear-sampled for the pre-immunoserum test. Hapten–BSA conjugates (500 μg) were injected each time. All injections were performed at multiple points. A program of five consecutive injections was carried out. For the two first injections separated by a 2-week period, the conjugate was dissolved in 1 mL PBS-complete Freund's adjuvant

(v–v). After the first two injections, three additional injections were performed in PBS-incomplete Freund adjuvant (v–v) at a 3-week interval. Five days after the fifth injection, a test was performed on a 5 mL blood sample to check the titers of the two immunsera. Fifty milliliters of the sample was collected 1 week after the test. Serum was obtained after blood clotting at 4 °C, for 24 h and centrifuged at 3000*g* for 10 min at 4 °C. Sera were stored at −20 °C in small aliquots. The efficiency of the immunization was tested in ELISA (as described below) by a direct binding of the antibody onto the coated conjugate.

4.11. Analysis of the antibody titer

These tests were performed with each of the antisera 4676 and 4677 as a direct revelation of the antibody binding for several dilutions to different concentrations of immobilized antigen. Coating of the microtitration plates was performed with the hapten–Thyr conjugates (200 μL/well) (as the immobilized antigen) in a carbonate buffer (0.05 M, pH 9.6) at 4 °C, overnight. Concentrations of coating-conjugates ranged from 10 μg/mL to 0.312 μg/mL with 1/2 dilution steps. The wells were then saturated with PBS-T-BSA-DMSO (PBS containing 1 mg/mL BSA, 0.05% Tween 20, and 1% DMSO) at 37 °C for 30 min. Plates were rinsed three times with PBS-T-DMSO (PBS, 0.05% Tween 20, 1% DMSO). Serial dilutions (1/1000; 1/2500; 1/5000; 1/10,000) of the specific antibodies were then added (200 μL/well). The incubation lasted for 2 h at 37 °C. The plates were washed three times with PBS-T-DMSO. Then 200 μL/well of the second antibody (swine anti-rabbit immunoglobulin coupled to Peroxidase from Dako, France) was added in PBS-T-BSA-DMSO. The incubation was performed at 37 °C for 30 min. To measure the peroxidase activity, 200 μL/well of substrate solution containing 0.005 M *o*-phenylenediamine (10 mg in 20 mL) and 0.00025% H_2O_2 30% (5 μL in 20 mL) in citrate-phosphate buffer (0.15 M, pH 5.0) was added. The reaction took place at room temperature for 30 min and was stopped with 50 μL/well of H_2SO_4 4 M. ODs were read at 490 nm.

4.12. ELISA procedure and analysis of the microbiological media

The ELISA procedure described here is a competition between a free compound and an immobilized antigen coated on the wells for the polyclonal antibody. According to the previous tests performed the following ELISA conditions were established. The 96-well ELISA plates (Greiner bio-one, Les Ulis, France) were coated overnight at 4 °C with hapten–Thyr conjugates at 0.25 μg/mL (200 μL/well) in carbonate buffer (0.05 M, pH 9.6). The wells were saturated with PBS-T-BSA-DMSO (PBS containing 1 mg/mL BSA, 0.05% Tween 20, and 1% DMSO) at 37 °C for 30 min and then washed three times with PBS-T-DMSO (PBS, 0.05% Tween 20, and 1% DMSO).

For the analysis of the microbiological media, an unknown solution disposition (100 μL/wells) together with 100 μL/well of antibody 4676 1/5000 dilution in PBS-T-BSA-DMSO were incubated for 2 h at 37 °C. The wells were washed three times with PBS-T-DMSO. The secondary antibodies (200 μL/well) in PBS-T-BSA-DMSO were added and incubated at 37 °C for 30 min. Revelation was carried out with 200 μL/well of substrate solution containing 0.005 M *o*-phenylenediamine and 0.00025% H_2O_2 30% in citrate-phosphate buffer (0.15 M, pH 5.0). The reaction was performed at room temperature for 30 min and was stopped with 50 μL/well of 4 M H_2SO_4. ODs were read at 490 nm. The interpretation of the data is performed taking into account the non-specific reactions measured in Blanks (obtained in the presence of the antibody but without the immobilized hapten) and with regard to the maximum of binding, namely Bo. Bo is obtained as in the titer test, by allowing the antibody to react onto the immobilized hapten–Thyr coated in the wells without any other compound added. When unknown samples are added they interfere with the immobilized antigen and fix a part of the antibodies available. Therefore, results are expressed as a percentage of maximum binding, that is (Bi − Blank)/(Bo − Blank), where Bi is the OD obtained when the unknown compound is added (Bo and Bi are means of homogenous duplicates performed twice, i.e., four independent determinations). In these conditions supernatants of bacterial cultures, semi-purified Amicoumacin B, and solutions of pure synthesized compounds (100 μg/mL) were tested.

Different *Bacillus* strains were selected in relation to our previous studies.[4,5] *B. subtilis* M1-2 and the probiotic strain BS3 were selected as Amicoumacin producing strains.[5] The *B. subtilis* 168 type strain and BM15 were selected as a negative control, because these strains do not produce Amicoumacins. Bacterial strains were cultured in Mueller Hinton (MH) starch broth (Difco Lab, Detroit, USA) at 30 °C during 72 h in an aerobic atmosphere as described previously.[5] Supernatants were obtained by centrifugation of the culture broth at 10,000 rpm during 10 min and serial dilutions effected in PBS buffer.

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Supplementary data

Supplementary material contains some details of the hapten–BSA conjugate characterization by UV-spectroscopy, SDS–PAGE and MALDI-TOF spectrometry as well as the experimental procedures and spectral characterization of synthetic parent compounds of Amicoumacins, which were used for cross-reactivity tests (Table 2, entries 10–13). Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.08.017.

References and notes

1. Shimojima, Y.; Shirai, T.; Baba, T.; Hayashi, H. *J. Med. Chem.* **1985**, *28*, 3.
2. Itoh, J.; Omoto, S.; Shomura, T.; Nishizawa, N.; Miyado, S.; Yuda, Y.; Shibata, U.; Inouye, S. *J. Antibiot. (Tokyo)* **1981**, *34*, 611.
3. Canedo Hernandez, L.; Acebal Sarabia, C.; Garcia Gravalos, D. Antitumor isocoumarins. US patent 5,925,671, 1996; WO 9627594; *Chem. Abstr.* **1996**, *125*, 273726.
4. Pinchuk, I. V.; Bressollier, P.; Verneuil, B.; Fenet, B.; Sorokulova, I. B.; Megraud, F.; Urdaci, M. C. *Antimicrob. Agents Chemother.* **2001**, *45*, 3156.
5. Pinchuk, I. V.; Bressollier, P.; Sorokulova, I. B.; Verneuil, B.; Urdaci, M. C. *Res. Microbiol.* **2002**, *153*, 269.
6. Itoh, J.; Shomura, T.; Omoto, S.; Miyado, S.; Yuda, Y.; Shibata, U.; Inouye, S. *Agric. Biol. Chem.* **1982**, *46*, 1255.
7. Shimojima, Y.; Hayashi, H.; Ooka, T.; Shibukawa, M. *Agric. Biol. Chem.* **1982**, *46*, 1823.
8. Shimojima, Y.; Hayashi, H.; Ooka, T.; Shibukawa, M.; Iitaka, Y. *Tetrahedron Lett.* **1982**, *23*, 5435.
9. Shimojima, Y.; Hayashi, H.; Ooka, T.; Shibukawa, M.; Iitaka, Y. *Tetrahedron* **1984**, *40*, 2519.
10. Hamada, Y.; Kawai, A.; Kohno, Y.; Hara, O.; Shiori, T. *J. Am. Chem. Soc.* **1989**, *111*, 1524.
11. Hamada, Y.; Hara, O.; Kawai, A.; Kohno, Y.; Shioiri, T. *Tetrahedron* **1991**, *47*, 8635.
12. Ward, R. A.; Procter, G. *Tetrahedron Lett.* **1992**, *33*, 3359.
13. Ward, R. A.; Procter, G. *Tetrahedron* **1995**, *51*, 12301.
14. Broady, S. D.; Rexhausen, J. E.; Thomas, E. J. *J. Chem. Soc., Chem. Commun.* **1991**, 708.
15. Broady, S. D.; Rexhausen, J. E.; Thomas, E. J. *J. Chem. Soc., Perkin Trans.* **1999**, *1*, 1083.
16. Kotsuki, H.; Araki, T.; Miyazaki, A.; Iwasaki, M.; Datta, P. K. *Org. Lett.* **1999**, *1*, 499.
17. Ghosh, A. K.; Bischoff, A.; Cappiello, J. *Org. Lett.* **2001**, *3*, 2677.
18. Ghosh, A. K.; Bischoff, A.; Cappiello, J. *Eur. J. Org. Chem.* **2003**, *5*, 821.
19. Superchi, S.; Minutolo, F.; Pini, D.; Salvadori, P. *J. Org. Chem.* **1996**, *61*, 3183.
20. Ghosh, A. K.; Cappiello, J. *Tetrahedron Lett.* **1998**, *39*, 8803.
21. Davies, G.; Russell, A. T. *Tetrahedron Lett.* **2002**, *43*, 8519.

22. Shinozaki, K.; Mizuno, K.; Wakamatsu, H.; Masaki, Y. *Chem. Pharm. Bull.* **1996**, *44*, 1823.
23. Mukai, C.; Miyakawa, M.; Hanaoka, M. *J. Chem. Soc., Perkin Trans. 1* **1997**, *6*, 913.
24. Kotsuki, H.; Miyazaki, A.; Ochi, M. *Tetrahedron Lett.* **1991**, *32*, 4503.
25. Wheeler, M. J. *Methods Mol. Biol.* **2006**, *324*, 1.
26. Shinkaruk, S.; Lamothe, V.; Schmitter, J.-M.; Fructus, A.; Sauvant, P.; Vergne, S.; Degueil, M.; Babin, P.; Bennetau, B.; Bennetau-Pelissero, C. *J. Agric. Food Chem.* **2008**, *56*, 6809.
27. Le Houerou, C.; Bennetau-Pelissero, C.; Lamothe, V.; Le Menn, F.; Babin, P.; Bennetau, B. *Tetrahedron* **2000**, *56*, 295.
28. Hauser, F. M.; Pogany, S. A. *Synthesis* **1980**, 814.
29. Kessar, S. V.; Singh, P.; Vohra, R.; Kaur, N. P.; Vanugopal, D. *J. Org. Chem.* **1992**, *57*, 6716.
30. Schlosser, M.; Geneste, H. *Tetrahedron* **1998**, *54*, 10119.
31. Bennetau, B.; Mortier, J.; Moyroud, J.; Guesnet, J. *J. Chem. Soc., Perkin Trans. 1* **1995**, *10*, 1265.
32. Mortier, J.; Moyroud, J.; Bennetau, B.; Cain, P. A. *J. Org. Chem.* **1994**, *59*, 4042.
33. Moyroud, J.; Guesnet, J.-L.; Bennetau, B.; Mortier, J. *Bull. Soc. Chim. Fr.* **1996**, *133*, 133.
34. Gohier, F.; Castanet, A.-S.; Mortier, J. *J. Org. Chem.* **2005**, *70*, 1501.
35. Nguyen, T.-H.; Chau, N. T. T.; Castanet, A.-S.; Nguyen, K. P. P.; Mortier, J. *Org. Lett.* **2005**, *7*, 2445.
36. Hauser, F. M.; Rhee, R. *J. Am. Chem. Soc.* **1977**, *99*, 4533.
37. Williams, D. R.; Barner, B. A.; Nishitani, K.; Phillips, J. G. *J. Am. Chem. Soc.* **1982**, *104*, 4708.
38. Thompson, R. C.; Kallmerten, J. *J. Org. Chem.* **1990**, *55*, 6076.
39. Roux, M.-C.; Paugam, R.; Rousseau, G. *J. Org. Chem.* **2001**, *66*, 4304.
40. Dondoni, A.; Perrone, D.; Merino, P. *J. Org. Chem.* **1995**, *60*, 8074.
41. Dieter Hoppe, T. H. *Angew. Chem. Int. Ed.* **1997**, *36*, 2282.
42. Tahara, N.; Fukuda, T.; Iwao, M. *Tetrahedron Lett.* **2002**, *43*, 9069.
43. Anderson, G. W.; Zimmerman, J. E.; Callahan, F. M. *J. Am. Chem. Soc.* **1964**, *86*, 1839.
44. Singh, K. V.; Kaur, J.; Varshney, G. C.; Raje, M.; Suri, C. R. *Bioconjug. Chem.* **2004**, *15*, 168.
45. Kanakam, C. C.; Mani, N. S.; Ramanathan, H.; Subba Rao, G. S. R. *J. Chem. Soc., Perkin Trans. 1* **1989**, *11*, 1907.
46. Smith, R. J.; Capaldi, R. A.; Muchmore, D.; Dahlquist, F. *Biochemistry* **1978**, *17*, 3719.