Organic & Biomolecular Chemistry

Cite this: *Org. Biomol. Chem.*, 2011, **9**, 5535

www.rsc.org/obc

PAPER

trans,*trans*-2,4-Hexadiene incorporation on enzymes for site-specific immobilization and fluorescent labeling†

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Received 14th March 2011, Accepted 26th April 2011
DOI: 10.1039/c1ob05401e

Lipase B from *Candida antarctica* (CAL-B) has been site-directedly modified by the introduction of a *trans*,*trans*-hexadiene moiety onto lipase molecules, identified by MALDI-TOF. This modification on CAL-B permitted its immobilization on Q-Sepharose supports in excellent yields (>95%) when native lipase was not immobilized at pH 7 and 25 °C. After the entire modification procedure, the catalytic activity of the protein on the solid support was surprisingly increased 2-fold. A tailor-made maleimide-fluorophore derivative was specifically covalently linked to the protein in high yield *via* a selective Diels–Alder reaction in aqueous media. Furthermore, the NBD-labeled-CAL-B was also immobilized on the ionic support, retaining around 80% of the specific activity. The preparation of this labeled-CAL-B was also possible by a Diels–Alder reaction on solid phase in excellent yields.

Introduction

The selective modification of proteins has been a long-standing challenge of modern chemical biology, with many applications, ranging from the study of natural post-translational modifications[1] to the development of protein-based materials. Site-specific modifications have been used in investigations of protein expression and localization, as tools in structure–function studies, in pharmacokinetics of protein-based drugs and to improve bioavailability, and in the development of biosensors.[2–4]

The incorporation of novel functionalities into proteins has been extensively studied for these items. Fluorescent labels, polymers, nucleic acids, polypeptides, and carbohydrates have been coupled to proteins to study or manipulate their function.[5–6] These modifications have also permitted the site-specific immobilization of proteins for purification, manipulation or property modulation.[7–8]

Importantly, these reactions occur at or near physiological pH and are compatible with the complex array of functional groups commonly found in biological macromolecules allowing conjugation reactions to be carried out on unprotected substrates.

In most of these cases, the side chain functionalities of the amino acids cysteine and lysine were targeted for conjugation reactions because of the reactivity of the free thiol and amine moieties, respectively.[9–10] However, cysteines are often involved in disulfide bonds that occur inside proteins. Breakage of these bonds can easily lead to loss of structural integrity and, hence, function. Lysine residues are positively charged, predominantly located at the protein surface, and relatively abundant. As a consequence, it is very likely that multiple functional handles are introduced when this residue is targeted for modification.[11] The properties of the bioconjugates thus generated are influenced by the degree and site of modification, therefore it is highly desirable to introduce new functional groups in a more defined manner.[12]

One approach to achieve this involves the modification of some protein amino acids of which only some residues are exposed to the solvent. Tyrosine functionalization by organometallic complexes[13] or chemical modifications of tryptophan[14] are two examples. Actually, different kinds of techniques for protein derivatization, based on synthetic organic chemistry such as expressed protein ligation,[15] Staudinger ligation[16] or "click" ligation,[17] have been developed in the context of biological molecules in aqueous media.

However, due to the multifunctionality of biomacromolecules, there is a major and continuing demand for development of new technology providing alternatives to the methods mentioned above. The required chemistry must be compatible with the functional groups found in proteins and proceed chemoselectively under mild conditions and in aqueous solution, preferably in the absence of any potentially denaturating co-solvent.

The chemical incorporation of a diene group is an interesting modification for site-specific introduction of different molecules into proteins such as fluorescent label, polymers, peptides, by chemoselective cycloaddition reaction such as the Diels–Alder [4π+2π] reaction. The characteristic of the Diels–Alder reaction makes it as an ideal methodology for bioconjugation[18–20] because it is accelerated in aqueous media at very mild reaction conditions (pH 6, room temperature). This reaction has been successfully applied for site-specific modification of streptavidin or Rab proteins.[21]

This chemical strategy could be quite interesting when applied to enzymes, in particular lipases whose properties can be

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c1ob05401e

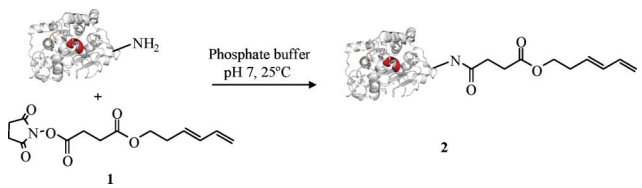
modulated to create artificial catalysts with new activities and high selectives.[22–23]

Therefore, in the present manuscript we describe the chemical modification of *Candida antarctica* (fraction B) lipase (CAL-B), one of the most used lipases in chemistry,[24] by a *trans-trans* hexadiene derivative for selective immobilization on ionic exchange matrices and for the direct or subsequent chemical incorporation of fluorescent labels by Diels–Alder reaction.

Results and discussion

Site-directed incorporation of *trans*,*trans*-2,4-hexadiene on CAL-B

The heterobifunctional cross-linker **1** (Scheme 1) was prepared as previously described[25] in two steps by esterification of succinic anhydride and hexadienol followed by transformation of the diene derivative into the *N*-hydroxysuccimidyl ester. This linker was attached to CAL-B molecules by acylation of *N* activated residues at pH 7 through the *N*-hydroxysuccinimidyl (NHS) group (Scheme 1). The molar ratio between the cross-linker and CAL-B was kept relatively low (3 : 1) to prevent multiple labeling of the lipase molecules.



Scheme 1 Site-specific modification of CAL-B by **1**.

However, analysis of the MALDI-TOF spectra of modified CAL-B (**2**) revealed that on average each lipase was equipped with three diene linkers (Fig. 1).

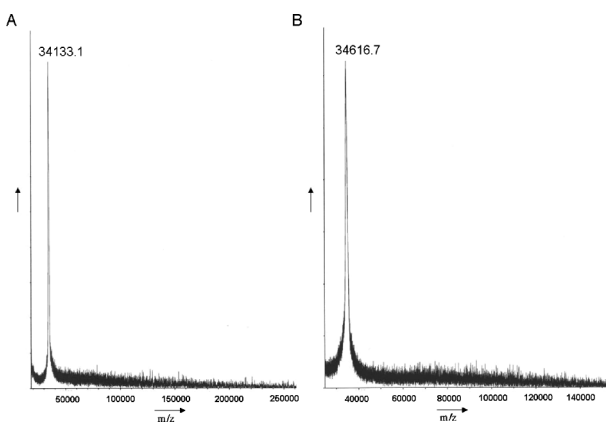


Fig. 1 MALDI-TOF spectra. A. CAL-B; B. diene–CAL-B (**2**).

Considering that lysines are not reactive at the pH of the modification (pK_a 10.2), the chemical modification of the protein was performed on different groups; for instance at the amino terminal residue and for example at the tryptophan moiety.[14]

Analyzing the tridimensional structure of CAL-B we observed that this lipase shows five tryptophans (Trp52, Trp65, Trp104, Trp113, Trp155) (Figure 1SI).

The profile of the UV-visible absorption spectrum for the soluble CAL-B and diene–CAL-B (**2**) was studied in a range of 199–500 nm. This experiment clearly showed the differences between CAL-B and modified-CAL-B (Fig. 2). The CAL-B spectrum showed three different peaks at 215, 238 and 260 nm. This broad peak at 260 nm, corresponding to the absorption bands of aromatic residues in the protein, was extremely decreased in the diene-modified CAL-B, indicating a loss on the aromaticity probably of the indole group in Trp by the chemical modification.[14]

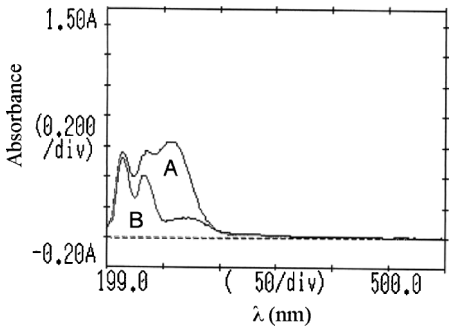


Fig. 2 UV-visible absorption spectra of modified-CAL-B. (A) CAL-B, (B) **2**.

Tryptic hydrolysis was performed to determine which amino acids were modified with diene molecules. A peptide profile from MALDI was obtained after the hydrolysis for CAL-B and diene modified CAL-B (**2**) (Fig. 3, Figures 2SI–3SI) corresponding approximately to 60% of the protein sequence (Fig. 4). Mainly, four different peptides were found (Fig. 3). A clear difference in the peptide sequence was found between these two enzymes.

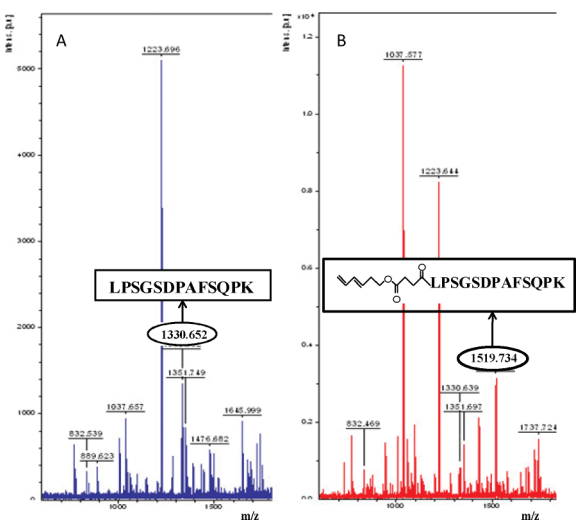


Fig. 3 MALDI spectra after tryptic hydrolysis. A. CAL-B. B. diene–CAL-B (**2**).

A peak at 1519.734 [$M_{\text{modified CAL-B}}$ + Na] was found corresponding to the peptide sequence LPSGSDPAFSQPK including the terminal amino group modified with the diene molecule (Fig. 3). The other peptides between both proteins were identical; the sequence included three tryptophans, Trp104, Trp113 and Trp 155 (Fig. 4), which were not modified.

Thus, by consideration of the loss in UV signal for modified CAL-B in Fig. 2 together with these results, we could estimate that

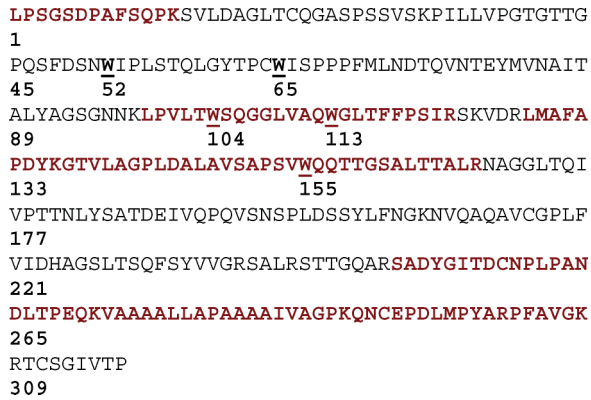


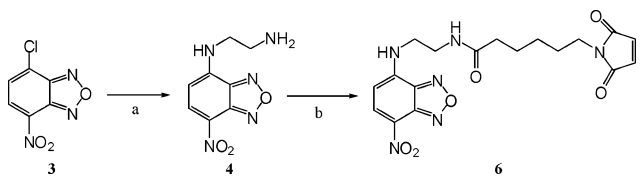
Fig. 4 Amino acid sequence of CAL-B. Peptides identified by tryptic hydrolysis in red.

CAL-B was probably modified in Trp52 and Trp65 in addition to the terminal amino group.

Site-directed incorporation of NBD labeled molecules on CAL-B by Diels–Alder cycloaddition in aqueous media

The modified-CAL-B (**2**), anchoring a water-stable diene, is an excellent candidate for further combination with a variety of maleimide probes to perform a Diels–Alder reaction in aqueous media. An interesting application of this classic synthetic organic reaction on proteins is to label them with fluorophores.[26]

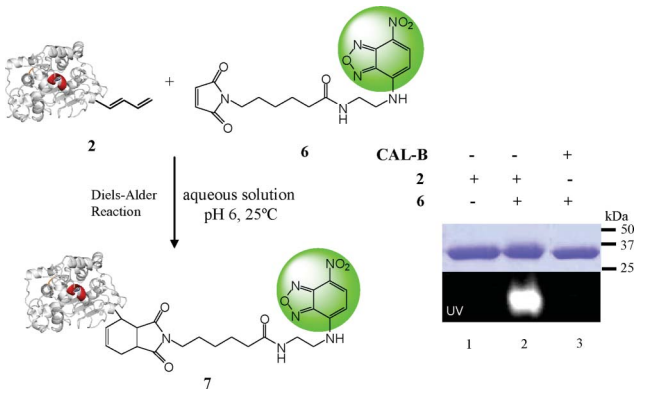
A 7-nitrobenzofurazan (NBD)-labeled maleimide **6** was previously synthesized (Scheme 2). NBD-Cl (**3**) was transformed on EDA-NBD **4** in 90% yield by a two step synthesis; an aromatic substitution with the previously synthesized *N*-Boc-1,2-diaminoethane[27] and a Boc deprotection by TFA treatment. After that, **4** was modified with 6-(maleimide)hexanoyl chloride (**5**) for the incorporation of the maleimide group on the NBD molecule. Compound **6** was synthesized in 77% overall yield after purification by flash chromatography.



Scheme 2 Synthesis of NBD-maleimide derivative **6**. a:1) $NH_2CH_2CH_2$-NHBoc, DIPEA, CH_2Cl_2; 2) TFA, CH_2Cl_2, r.t.; b: **5**, DIPEA, CH_2Cl_2, r.t.

The protein–hexadiene complex **2** was treated with fluorescent NBD-labeled maleimide derivative **6** (30-fold excess) in 5 mM sodium phosphate buffer at pH 6.5 and room temperature for 24 h (Scheme 3). Subsequently unreacted dienophile was removed by membrane centrifugation affording fluorescent protein complex **7**. The formation of this new fluorescent protein was verified by SDS-PAGE *via* UV-detection (Scheme 3). Incubation of unmodified CAL-B and dienophile molecule **6** resulted in no detectable specific product (Scheme 3), confirming that the observed conjugation of maleimide probes proceeded *via* Diels–Alder ligation.

Therefore these results revealed that the Diels–Alder reaction is an interesting methodology for chemical modification of lipases,



Scheme 3 Maleimide–NBD derivative **6** incorporation in CAL-B **2** molecules by Diels–Alder conjugation.

compatible with reactive amino acids such as lysines, aspartic acids, glutamic acids or histidines.

Immobilization of modified-CAL-B (2, 7)

The stability of the hexadiene function in aqueous solution and its compatibility with the functional groups present in amino acids opens up the opportunity to combine the Diels–Alder ligation method with other techniques, for example the oriented immobilization of enzymes.

Particularly, the immobilization of proteins by ionic exchange on cationic supports goes through a multipoint mechanism, where the enzyme is oriented by the richest area of carboxylic groups on the protein surface.[28] This is an interesting methodology because represents a reversible, although strong, immobilization methodology.[28]

This characteristic permits us to work with the proteins on the solid-phase, for example for further modifications, and finally recovery by a simple desorption with salt.

However, commercial CAL-B was not immobilized on DEAE or Q-Sepharose – supports with a high density of tertiary or quaternary amines respectively – at pH 7 and 25 °C. Increasing pH up to 9 did not improve the result.

The diene–CAL-B **2** was successfully immobilized by ionic exchange on these supports at these mild conditions. More than 95% yield of immobilized protein was achieved in one hour even using 1 mg up to 12 mg pure protein per g support (Fig. 5A). To demonstrate that the immobilization is exclusively through ionic interactions, the immobilized enzyme preparation was incubated at different concentrations of NaCl (Figure 4SI). A full desorption of the protein from the support was found at 1 M of NaCl, indicating that the enzyme was strongly immobilized on the resin.

Surprisingly, the specific activity of the modified enzyme in the hydrolysis of pNPB was increased 2 fold after the immobilization (Fig. 5B) which never has been observed for a lipase in an ionic exchange interaction, a characteristic only observed for this enzyme on the hydrophobic interfaces.[29] Modified CAL-B **2** showed the same specific activity as native CAL-B in solution. The conformational change of Phe48 (Figure 4SI) – after the chemical modification of the protein with the diene – could be responsible for this enzyme immobilization or hyperactivation.

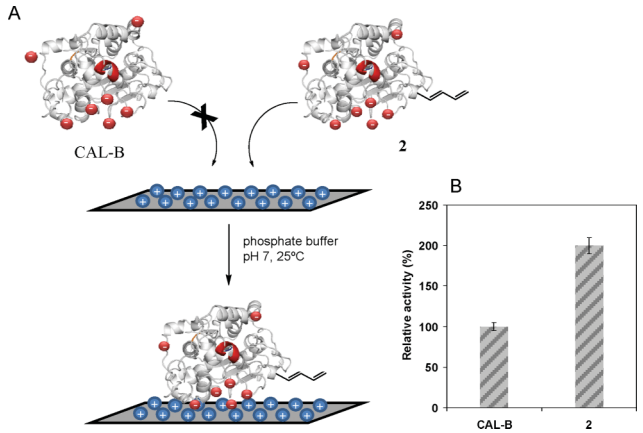


Fig. 5 Immobilization of CAL-B on Q-Sepharose supports. A) Immobilization scheme. B) Relative specific activity of unmodified CAL-B and diene CAL-B **2**. Specific activity was measured on the hydrolysis of pNPB (see experimental part).

The oriented immobilization of the labeled-CAL-B (**7**) on Q-Sepharose support is shown in Fig. 6. The adduct **7** was immobilized on this support at pH 7 and 25 °C.

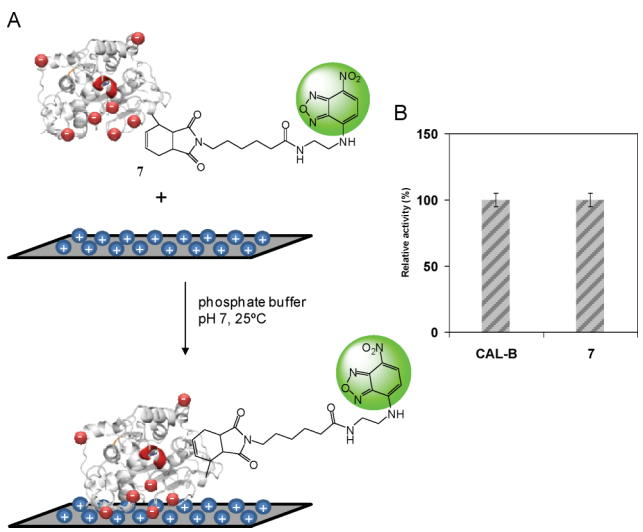


Fig. 6 Immobilization of labeled-CAL-B **7** on Q-Sepharose supports. A) Immobilization scheme. B) Relative specific activity of unmodified CAL-B and diene CAL-B **7**. Specific activity was measured on the hydrolysis of pNPB (see experimental section).

In this case, 80% of the offered protein (3 mg g^{-1} support) was immobilized after 1 h although no hyperactivation of the modified enzyme was observed after immobilization, the specific activity of the immobilized **7** was similar to that in solution and even similar to native CAL-B.

Labeling of diene–CAL-B (2) on the solid-phase

The site-specific incorporation of the fluorophore on the CAL-B was performed on the solid phase too. The hyperactivated and immobilized CAL-B diene was treated with NBD-labeled maleimide **6** (60-fold excess) in 5 mM sodium phosphate buffer at pH 6.5 and room temperature for 48 h (Fig. 7). Then, the

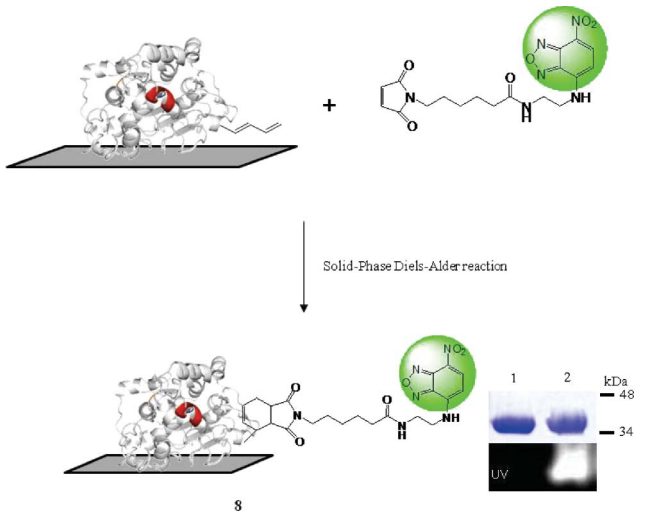


Fig. 7 Fluorescent labeling of immobilized CAL-B **2** by Diels–Alder. SDS-PAGE: Lane 1: CAL-B **2**, lane 2: protein supernatant from the boiled preparation **8**.

unreacted dienophile was removed by simple filtration and several washing steps using distilled water.

Finally, 100 mg of CAL-B- Q-Sepharose derivative **8** (Fig. 7) were treated with 100 µL of rupture buffer and boiled for 5 min. Then 12 µL of the solution were rapidly injected on the SDS-PAGE gel and the Diels–Alder ligation was analyzed *via* UV-detection (Fig. 7). Therefore, the site-directed incorporation of NBD was possible on the lipase on the solid-phase.

Conclusions

A convenient method to modify CAL-B in a site-specific way has been developed. A *trans*,*trans*-hexadiene moiety was introduced by modification of the amino terminal residue and tryptophans on the lipase molecules to act as a functional handle. Subsequently, a tailor-made fluorophore was covalently linked *via* the highly selective Diels–Alder reaction in aqueous media. This modification procedure on CAL-B permitted its immobilization on Q-Sepharose supports in excellent yields (>95%) when native lipase was not immobilized at pH 7 and 25 °C. After the entire modification procedure, catalytic activity was surprisingly increased 2-fold once immobilized on the solid ionic support.

Finally, the labeled-CAL-B also was immobilized on this support although retaining the same initial activity and the incorporation of the fluorophore was also successfully performed directly on the immobilized modified enzyme. This solid phase method presents many advantages for an easy coupling and purification with high final yields.

In particular, the site-specific incorporation of fluorophores in lipases could be useful for studying the catalytic mechanism by controlling the opening and closing of the lid for example using a FRET system,[30] the enzyme dynamics at different interfaces,[31] to quantify surface diffusion by fluorescence recovery after photobleaching (FRAP) [31] or for binding and characterization on surfaces, such as magnetic nanoparticles.[32]

Experimental

Q®-Sepharose 4BCL was from GE Healthcare (Uppsala, Sweden). *trans*,*trans*-2,4-Hexadienol, *p*-nitrophenyl butyrate (*p*NPB), succinic anhydride, *N*-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), 4-chloro-7-nitrobenzofurazan (NBD-Cl), *N*,*N*-diisopropylethylamine ethylenediamine (DIPEA), ethylenediamine (EDA) and 6-maleimidohexanoic acid were purchased from Sigma Chem Co. *Candida antarctica* lipase (fraction B) (CAL-B) was purchased from Novozymes. MALDI MS spectra were recorded by using 2,5-dihydroxybenzoic acid (DHB) or α-cyano-4-hydroxy cinnamic acid (CHCA) as matrix. The tryptic hydrolysis and the protein identification by LC-MS/MS were carried out in the 'CBMSO PROTEIN CHEMISTRY FACILITY', a member of the ProteoRed network.

6-(Maleimido)-*N*-(2-(7-nitrobenzo[*c*][1,2,5]oxadiazol-4-ylamino)ethyl)hexanamide (6)

NBD-Cl (**3**) (0.25 g, 1.245 mmol) was added at room temperature to a solution of *N*-Boc-1,2-diaminoethane (0.2 g, 1.245 mmol) and *N*,*N*-diisopropylethylamine (0.434 ml, 2.49 mmol) in CH_2Cl_2 (10 mL). The resulting dark brown solution was stirred until disappearance of the starting products monitored by TLC (ethyl acetate : n-hexane, 6 : 4). After 3 h the solution was diluted with 10 mL of CH_2Cl_2 and the organic phase was washed with 3 × 10 mL of $NaHCO_3$ 5% in distilled water and 2 × 10 mL with water. The recollected organic phase was dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. Subsequently, the obtained crude was dissolved in 5 mL of CH_2Cl_2 and then 5 mL of trifluoroacetic acid were slowly added dropwise at room temperature. After 1 h, diethyl ether (60 ml) was added. The precipitated product was collected by vacuum filtration, washed with cold diethyl ether and dried, yielding **4** (90%).

6-(Maleimido)hexanoic acid chloride (**5**) was prepared by adding dropwise oxalyl chloride (4.73 mmol, 0.4 mL) to an ice-bath cooled solution of maleimidocaproic acid (0.946 mmol, 0.2 g) and 10 μL of anhydrous DMF in CH_2Cl_2 (5 mL). After a few minutes, the mixture is allowed to reach room temperature and left under stirring over night. Volatiles were removed under reduced pressure and the residue was azeotroped three times with 5 ml of CH_2Cl_2 each time. The resulting yellow crude residue was used without any further purification.

Aminated-NBD **4** (0.41 mmol, 0.138 g) and *N*,*N*-diisopropylethylamine (1.61 mmol, 0.285 mL) were suspended in anhydrous CH_2Cl_2 (4 mL) and the mixture was cooled on an ice bath. After 10 min, to this mixture, the acyl chloride **5** (0.451 mmol, 0.094 g dissolved in 1 mL of CH_2Cl_2) was added dropwise. After 15 min on ice, the reaction mixture was allowed to warm up to room temperature and stirring over night.

The solution was concentrated under reduced pressure and the residue was purified by flash column chromatography on silica gel (CH_2Cl_2/acetone: 7/3) to give a bright orange solid **6** (0.4 g, overall yield of entire synthetic pathway 77%). ^{1}H-NMR ($CDCl_3$, 400 MHz): δ = 8.48 (d, 1H, J = 8.62, NBD), 7.7 (dd, 1H, J = 8.98, J = 3.27, NH), 7.53 (dd, 1H, J = 9.0, J = 3.3, NH), 6.68 (s, 2H, maleimide), 6.16 (d, 1H, J = 8.63, NBD), 3.73–3.59 (2 m, 4H, 2CH_2-EDA), 3.48 (t, 2H, J = 7.10, CH_2 next to maleimide), 2.25 (t, 2H, J = 7.35, CH_2 next to carbonyl), 1.74–1.64 (m, 4H, maleimide-2CH_2), 1.45–1.37 (m, 2H, maleimide-CH_2). HRMS (ESI+): m/z calcd for $C_{18}H_{20}N_6O_6$ $[M + H]^+$ 417.39, found 417.15.

Preparation of the CAL-B–diene conjugate (2)

To a solution of commercial CAL-B (50 mg, 1.43 nmol) in 5% dioxane in 5 mM NaH_2PO_4 pH 6.5 buffer solution, the diene cross-linker **1** (4.3 nmol in 500 μl of dioxane) was added and kept stirring overnight at 25 °C. The reaction mixture solution was dialyzed with four changes of distilled water. After the samples were concentrated and characterized by MALDI-TOF.

Enzymatic activity assay

The activities of the soluble lipase, supernatant and enzyme suspension were analyzed spectrophotometrically measuring the increment in absorbance at 348 nm produced by the release of *p*-nitrophenol (*p*NP) (ε = 5.150 M^{-1} cm^{-1}) in the hydrolysis of 0.4 mM *p*NPB in 25 mM sodium phosphate at pH 7 and 25 °C. To initialize the reaction, 0.05–0.2 mL of lipase solution or suspension was added to 2.5 mL of substrate solution in magnetic stirring. Enzymatic activity is given as μmol of hydrolyzed *p*NPB per minute per mg of enzyme (IU) under the conditions described above.

Diels–Alder ligation of the soluble CALB–diene conjugate 2 with fluorescently labeled dienophile probe (6)

Hexadiene-conjugate **2** at 0.1 mg mL^{-1} concentration in 5 mM NaH_2PO_4 pH 6.5 buffer was incubated with 30-fold excess of maleimide probe **6** (dissolved in 500 μL of acetonitrile). The temperature was kept at 25 °C for 24 h while shaking. After this time, the excess dienophile was removed by passing the reaction mixture through an Amicon Ultra 10kDa (DyeEx columns from Qiagen) and several times washed with 5% (v/v) acetonitrile in distilled water. The ligated protein **7** was analyzed by SDS-PAGE on 10% (w/v) polyacrylamide gels by Coomassie staining. To detect coupling of fluorescent probe **6** to hexadiene–CAL-B, prior of the Coomassie staining, the gel was analyzed using a Molecular Imager Gel Doc XR System - Bio-Rad Laboratories.

Immobilization of CALB–diene (2) and CAL-B–NBD (7) on Q Sepharose support

One gram of Q-Sepharose was added to 30 mL of modified protein solution (3 mg CALB–diene **2** or CALB–NBD **7** dissolved in 5mM NaH_2PO_4 buffer pH 7). The suspension was then stirred for 1 h at 25 °C. Periodically, samples of the supernatants and suspensions were withdrawn, and the enzyme activity was measured as described above. After that, the immobilization was stopped, the supernatant removed by filtration and the supported modified lipase washed several times with distilled water. Immobilization yield for CAL-B–diene was >95% and for CAL-B–NBD was 70%.

Solid-phase Diels–Alder ligation of the supported CAL-B–diene conjugate with fluorescently labeled dienophile probe 6

CALB-diene immobilized in Sepharose Q (3 $mg_{protein}$ g^{-1}, 0.08571 nmol) was suspended in 20 ml of 5 mM NaH_2PO_4 pH 6.5 buffer. 60-fold excess of NBD-maleimide probe **6** (2.14 mg,

5.1425 nmol dissolved in 500 μL of acetonitrile) were added. The temperature was kept at 25 °C for 48 h while shaking. After this time, the reaction was stopped and the derivative (supported lipase modified with fluorophore) recovered by filtration and washing several times with distilled water.

Acknowledgements

This work has been sponsored by the Spanish Ministry of Science and Innovation (Project CTQ2009-07568) and CSIC (Intramural Project 200980I133). We also thank to CONICYT and Programa Bicentenario Becas-Chile for financial support for Mr. Romero. We thank Dr Valeria Grazu (Institute of Nanotechnology, University of Zaragoza) for MALDI analytical support.

Notes and references

1 (*a*) D. P. Gamblin, S. I. van Kasteren, J. M. Chalker and B. G. Davis, *FEBS J.*, 2008, **275**, 1949; (*b*) G. Walsh and R. Jefferis, *Nat. Biotechnol.*, 2006, **24**, 1241.
2 L. Wang, L. Harrington, E. Trebicka, H. N. Shi, J. C. Kagan, C. C. Hong, H. Y. Lin, J. L. Babitt and B. J. Cherayil, *J. Clin. Invest.*, 2009, **119**, 3322.
3 N.-N. Aye-Han, Q. Ni and J. Zhang, *Curr. Opin. Chem. Biol.*, 2009, **13**, 392–397.
4 L. Tang, A. M. Persky, G. Hochhaus and B. Meibohm, *J. Pharm. Sci.*, 2004, **93**, 2184.
5 (*a*) W. P. Heal and E. W. Tate, *Org. Biomol. Chem.*, 2010, **8**, 731; (*b*) M. Sunbul and J. Yin, *Org. Biomol. Chem.*, 2009, **7**, 3361; (*c*) K. N. Allen and B. Imperiali, *Curr. Opin. Chem. Biol.*, 2010, **14**, 247.
6 (*a*) H. Y. Song, M. H. Ngai, Z. Y. Song, P. A. MacAry, J. Hobley and M. J. Lear, *Org. Biomol. Chem.*, 2009, **7**, 3400; (*b*) M. A. Gauthier and H.-A. Klok, *Chem. Commun.*, 2008, 2591; (*c*) E. Baslé, N. Joubert and M. Pucheault, *Chem. Biol.*, 2010, **17**, 213.
7 (*a*) F. Rusmini, Z. Zhong and J. Feijen, *Biomacromolecules*, 2007, **8**, 1775; (*b*) P. Jonkheijm, D. Weinrich, H. Schroder, C. M. Niemeyer and H. Waldmann, *Angew. Chem., Int. Ed.*, 2008, **47**, 9618; (*c*) D. Weinrich, P.-C. Lin, P. Jonkheijm, U. T. T. Nguyen, H. Schröder, C. M. Niemeyer, K. Alexandrov, R. Goody and H. Waldmann, *Angew. Chem., Int. Ed.*, 2010, **49**, 1252.
8 (*a*) L. S. Wong, F. Khan and J. Micklefield, *Chem. Rev.*, 2009, **109**, 4025; (*b*) M. Shi, J. H. Wosnick, K. Ho, A. Keating and M. S. Shoichet, *Angew. Chem., Int. Ed.*, 2007, **46**, 6126.
9 K. Blank, J. Morfill and H. E. Gaub, *ChemBioChem*, 2006, **7**, 1349.
10 F. M. Veronese, B. Sacca, P. P. de Laureto, M. Sergi, P. Caliceti, O. Schiavon and P. Orsolini, *Bioconjugate Chem.*, 2001, **12**, 62.
11 Y. Wang, S. Youngster, M. Grace, J. Bausch, R. Bordens and D. F. Wyss, *Adv. Drug Delivery Rev.*, 2002, **54**, 547.
12 K. L. Heredia, D. Bontempo, T. Ly, T. J. Byers, S. Halstenberg and H. D. Maynard, *J. Am. Chem. Soc.*, 2005, **127**, 16955.
13 S. D. Tilley and M. B. Francis, *J. Am. Chem. Soc.*, 2006, **128**, 1080.
14 (*a*) J. M. Antos, J. M. McFarland, A. T. Iavarone and M. B. Francis, *J. Am. Chem. Soc.*, 2009, **131**, 6301; (*b*) J. M. Antos and M. B. Francis, *J. Am. Chem. Soc.*, 2004, **126**, 10256; (*c*) H. Chiba, M. Hisatake, M. Hirose and E. Sugimoto, *Biochim. Biophys. Acta (BBA)-Enzym.*, 1973, **327**, 380.
15 (*a*) T. W. Muir, *Annu. Rev. Biochem.*, 2003, **72**, 249; (*b*) V. Muralidharan and T. W. Muir, *Nat. Methods*, 2006, **3**, 429.
16 J. Kalia, N. L. Abbott and R. T. Raines, *Bioconjugate Chem.*, 2007, **18**, 1064.
17 (*a*) B. Wieczorek, B. Lemcke, H. P. Dijkstra, M. R. Egmond, R. J. M. Klein Gebbink and G. Van Koten, *Eur. J. Inorg. Chem.*, 2010, **13**, 1929; N. D. Weikart and H. D. Mootz, *ChemBioChem*, 2010, **11**, 774; K. A. Kalesh, H. Shi, J. Ge and S. Q. Yao, *Org. Biomol. Chem.*, 2010, **8**, 1749; S. K. Mamidyala and M. G. Finn, *Chem. Soc. Rev.*, 2010, **39**, 1252.
18 M. Wiessler, W. Waldeck, C. Kliem, R. Pipkorn and K. Braun, *Int. J. Med. Sci.*, 2010, **7**, 19.
19 J. M. Langenhan and J. S. Thorson, *Curr. Org. Synth.*, 2005, **2**, 59.
20 T. K. Tiefenbrunn and P. E. Dawson, *Biopolymers*, 2010, **94**, 95.
21 J. M. Palomo, *Eur. J. Org. Chem.*, 2010, **33**, 6303.
22 J. M. Palomo, *Curr. Org. Synth.*, 2009, **6**, 1–14.
23 (*a*) C. Godoy, B. de las Rivas, M. Filice, G. Fernández-Lorente, J. M. Guisan and J. M. Palomo, *Process Biochem.*, 2010, **45**, 534–541; (*b*) J. M Palomo, G. Fernández-Lorente, R. Fernández-Lafuente and J. M. Guisán, *Adv. Synth. Catal.*, 2007, **349**, 1119–1127.
24 T. Hudlicky and J. W. Reed, *Chem. Soc. Rev.*, 2009, **38**, 3117.
25 A. D. de Araujo, J. M. Palomo, J. Cramer, O. Seitz, K. Alexandrov and H. Waldmann, *Chem.–Eur. J.*, 2006, **12**, 6095.
26 (*a*) A. D. de Araujo, J. M. Palomo, J. Cramer, M. Kohn, H. Schroder, R. Wacker, C. Niemeyer, K. Alexandrov and H. Waldmann, *Angew. Chem., Int. Ed.*, 2006, **45**, 296; (*b*) A. Doerr, *Nat. Methods*, 2006, **3**, 77.
27 A. Eisenführ, P. S. Arora, G. Sengle, L. R. Takaoka, J. S. Nowick and M. Famulok, *Bioorg. Med. Chem.*, 2003, **11**, 235.
28 R. Torres, C. Mateo, M. Fuentes, J. M. Palomo, C. Ortiz, R. Fernández-Lafuente, J. M. Guisan, A. Tam and M. Daminati, *Biotechnol. Prog.*, 2002, **18**, 1221.
29 U. Derewenda, A. M. Brzozowski, D. M. Lawson and Z. S. Derewenda, *Biochemistry*, 1992, **31**, 1532.
30 (*a*) K. Kikuchi, *Chem. Soc. Rev.*, 2010, **39**, 2048; (*b*) G. R. Szilvay, M. A. Blenner, O. Shur, D. M. Cropek and S. Banta, *Biochemistry*, 2009, **48**, 11273.
31 (*a*) A. W. Sonesson, H. Brismar, T. H. Callisen and U. M. Elofsson, *Langmuir*, 2007, **23**, 2706; (*b*) L. J. Cruz, P. J. Tacken, F. Bonetto, S. I. Buschow, H. J. Croes, M. Wijers, I. J. de Vries and C. G. Figdor, *Mol. Pharmaceutics*, 2011, **8**, 520.
32 B. E. Feller, J. T. Kellis, L. G. Cascao-Pereira, C. R. Robertson and C. W. Frank, *Langmuir*, 2010, **26**, 18916.

