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COMMUNICATION

Unexpected enzyme-catalyzed regioselective acylation of flavonoid aglycones and rapid product screening†

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Unprecedented regioselective acylation of flavonoid aglycones was achieved using *Candida antarctica* lipase B (CALB). The rapid screening of product formation was performed by the use of the high resolution phenol-type OH ^{1}H NMR spectral region recorded after the addition of picric acid.

Natural products and especially plant-derived polyphenols cover a very interesting chemical space of biological relevance due to their vast chemical diversity, and fine-tuning for optimal interactions with biological macromolecules through evolutionary selection.[1] Indeed, they present numerous anti-inflammatory, anti-angiogenic and anti-carcinogenic effects in cell culture and in animal models[2,3] with more than 50% of the current effective drugs in the cancer chemotherapy area originating from natural products.[4,5] Although phenol-type OH groups constitute the most important and frequently encountered chemotypes conferring bioactivity, recent studies revealed that protection of these functional groups by *O*-methylation or *O*-acetylation resulted in analogues with higher intestinal absorption, resistance to hepatic metabolism and better anticancer activity in respect to the original compounds.[6,7] Thus, such protection is highly desirable but not in a form of generic transformation of the original molecule but rather in a regioselective way.[8,9] To achieve this and to avoid the tedious protection/deprotection steps required in a classical chemical synthesis method, due to the numerous reactive hydroxyl groups of polyphenols, we hypothesized that biocatalysis could be a powerful tool. *Candida antarctica* lipase B (CALB) is an excellent biocatalyst for asymmetric organic chemistry with high enantioselectivity and regioselectivity in a broad range of substrates.[10–12] CALB, although it has been reported to acylate glycosylated flavonoids,[13–17] has been determined to have low activity for a range of tertiary alcohols and polyphenol aglycones[15,18–20] and no detectable activity in flavonoids aglycones.[14,15,21–23]

In contrast to the current perception that CALB is incapable to acylate flavonoid aglycones, herein we describe for the first time the enzymatic acylation of the phenolic group of flavonoid aglycones (quercetin and naringenin) by transesterification with vinyl acetate or vinyl butyrate in organic solvents.

Factors which affect the biocatalytic process such as the acyl donor and organic solvent were determined. Product formation was rapidly monitored through ^{1}H NMR by harnessing the great shielding sensitivity of the phenol-type OH absorptions.[24,25] Spectral resolution enhancement, for a factor of over 500, was achieved after the addition of picric acid to the reaction products. The resonance absorption of the OH(5) protons of flavonoid aglycones could be used as an internal sensor to monitor the transformation efficacy and regioselectivity. This is a novel and rapid method for screening product formation in enzyme-mediated polyphenol aglycones transformations without the use of tedious and time consuming isolation techniques.

Our initial flexible docking calculations[26–28] indicated that the flavonoid quercetin could be favourably accommodated for transformation in the substrate binding site of CALB and adopt a preferable orientation with its 3′ hydroxyl group facing the catalytic active site (Fig. 1). To probe the *in silico* observations (Fig. 1), the flavonoid quercetin, which presents numerous biological activities, was chosen as the model system for such catalysis (Scheme 1). Indeed, product formation was observed (45% yield) only in the presence of the enzyme (ESI, Fig. S1†), and optimum reaction conditions (solvent, acyl donor) were determined (ESI, Fig. S2†). Reaction products (t_R = 14.30 min) can

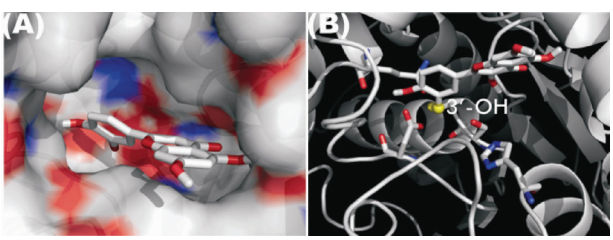

Fig. 1 Three dimensional model of quercetin bound to the active site of lipase B as derived from flexible docking calculations. Quercetin is shown as sticks and CALB is shown (A) as surface and (B) as ribbons. In (B) the 3′-OH of quercetin is illustrated as a yellow sphere and the catalytic amino acids as sticks.

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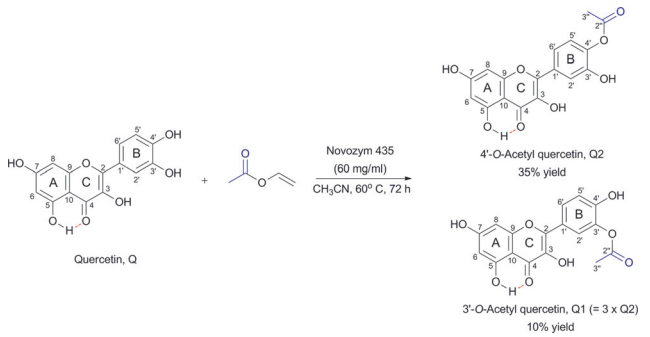

Scheme 1 Flavonoids are comprised of two benzene rings (A and B) linked through a heterocyclic pyran or pyrone ring (C) in the middle. The OH(5) proton is implicated in the formation of an intramolecular hydrogen bond that is depicted with a red line. Acylation of quercetin can, in principle, take place in positions 3, 5, 7, 3′ and 4′. CALB (Novozyme 435) was found to regioselectively acylate quercetin in ring B leading to two products, Q1 and Q2. Q2 was formed with 3 times higher yield. The reaction was performed with quercetin (3 μmol), vinyl acetate (1 mmol) and 60 mg ml^{-1} of catalyst in solvent (200 μl) at 60° C.

be readily separated from unreacted quercetin (t_R = 12.50 min) through preparative HPLC. Although acylation of quercetin can take place in five different positions (5, 7, 3, 3′ and 4′), ESI-MS analysis confirmed that only mono-acylation took place (ESI, Fig. S7†). In order to unambiguously determine the derived acylation products, ^{1}H NMR experiments were performed.

Fig. 2A illustrates selected regions of the ^{1}H NMR spectrum in DMSO-d_6 of the reaction product(s). A strongly deshielded asymmetric broad resonance was observed at 12.5 ppm. This resonance can be assigned to the OH(5) protons of flavonoids, located in the benzene ring A (Scheme 1), participating in strong intramolecular hydrogen bonding with the carbonyl group

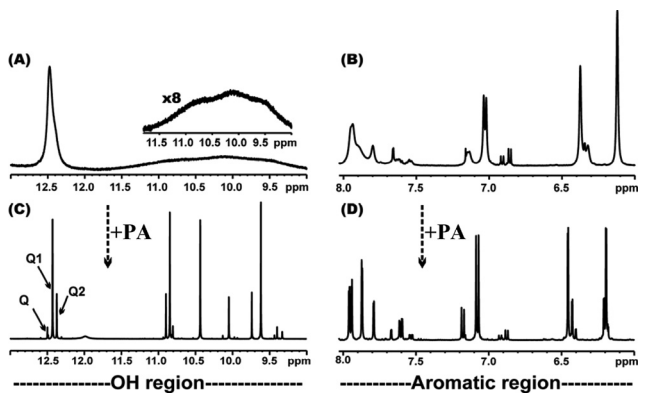

Fig. 2 500 MHz 1D ^{1}H NMR spectrum of the OH and aromatic regions of the enzymatically obtained monoacylated derivatives of quercetin (1.35 μmol) in 500 μl DMSO-d6. The –OH and aromatic spectral regions are illustrated in (A), (B) before and in (C), (D) after the addition of 25.30 nmol of picric acid (2.5 μl from a 10.18 mM stock solution), respectively. The molar ratio of picric acid and acetyl quercetin derivatives in the reaction was 1 : 53. Picric acid (PA) resulted in a spectral resolution enhancement by a factor of over 560. In (A) the inset shows a zoom-in (×8) on the spectral region from 9 to 11 ppm. In (C) the absorptions of the OH(5) protons for Q (unreacted quercetin), Q1 and Q2 (the retrieved derivatives) are indicated with arrows and could be used as internal sensor for monitoring the reaction products.

CO(4). In the region from 9 to 11 ppm, where the OH(3′), OH(4′), OH(3) and OH(7) protons of flavonoids were expected, very broad resonance absorptions were observed with a composite linewidth ($\Delta\nu_{1/2} \approx 874$ Hz). Similarly, in the spectral region from 6 to 8 ppm (Fig. 2B), where the aromatic resonances of flavonoids are expected to absorb, very broad peaks were observed. Therefore, the low quality and complexity of the recorded spectrum prohibited a detailed spectral assignment of the reaction products. Interestingly, not only resonances of the exchangeable –OH protons but also the aromatic region suffered from severe line broadening, possibly due to proton exchange and/or aggregation phenomena (Fig. 2B).

In order to surmount such problems we used a novel methodology which can enhance spectra quality. Specifically, the reaction products were titrated with picric acid. Picric acid (PA) has a very low pK_a value (0.42), in comparison to the low acidity of quercetin (p$K_a \approx 7.70$), that could fine-tune the proton chemical exchange and disrupt hydrogen bonding networks of polyphenols. In addition, since picric acid has been employed in numerous interactions such as electrostatic, hydrogen bonding[29] and π–π stacking interactions[30] it can intercalate with aggregates formed between stacked aromatic rings, thus disrupting potential forms of aggregation (ESI, Fig. S3†).

Indeed, after addition of picric acid (25.30 nmol) the recorded ^{1}H NMR spectrum was significantly improved not only in the –OH region (Fig. 2C) but also in the aromatic region (Fig. 2D). Specifically, the broad peak at 12.48 ppm with a composite asymmetric linewidth ($\Delta\nu_{1/2} \approx 43.6$ Hz), after the addition of picric acid, was resolved into three discrete and very sharp resonances at 12.37 ppm ($\Delta\nu_{1/2} \approx 1.4$ Hz), 12.43 ppm ($\Delta\nu_{1/2} \approx 1.5$ Hz) and 12.50 ppm ($\Delta\nu_{1/2} \approx 1.6$ Hz). The resonance at 12.50 ppm should be attributed to the OH(5) of unreacted quercetin. The other two resonances are due to the OH(5) protons of two monoacylated products. In the spectral region of 9 to 11 ppm, nine sharp resonance absorptions appeared, with $\Delta\nu_{1/2} \approx 1.6 \pm 0.4$ Hz, after the addition of picric acid, which previously appeared as extremely broad composite resonances ($\Delta\nu_{1/2} \approx 874$ Hz). Therefore, with the addition of picric acid a great reduction in the linewidth by a factor of over 560 was achieved. This enhanced spectral refinement was also evident in the aromatic spectral region. For instance, the broad resonance absorption at 6.12 ppm ($\Delta\nu_{1/2} \approx 6.2$ Hz) resulted in three high resolution resonances at 6.18 ppm, 6.19 ppm and 6.21 ppm (Fig. 2D).

This high resolution spectrum indicated the presence of more than a single species in the reaction products. In order to quantitate the different chemical species in the reaction products, NMR diffusion experiments (DOSY) were performed.[31] The DOSY spectra clearly suggested the presence of two reaction products (data not shown).

Application of 2D ^{1}H–^{13}C HMBC and ^{1}H–^{13}C HSQC (Fig. 3) of the same solution as in Fig. 2(C) resulted in a significant number of cross peaks of the –OH groups. Several long range connectivities of $^{2-4}J(^1H,^{13}C)$ couplings were observed which allowed the complete assignment of the carbon skeleton of the monoacylated derivatives of quercetin in Scheme 1 (see the ESI, Tables S2–S3 and Fig. S11–S14 for the complete assignment of the two products†). More specifically, acylation took place at the 4′ (OH(4′)) or the 3′ (OH(3′)) position of quercetin (located in

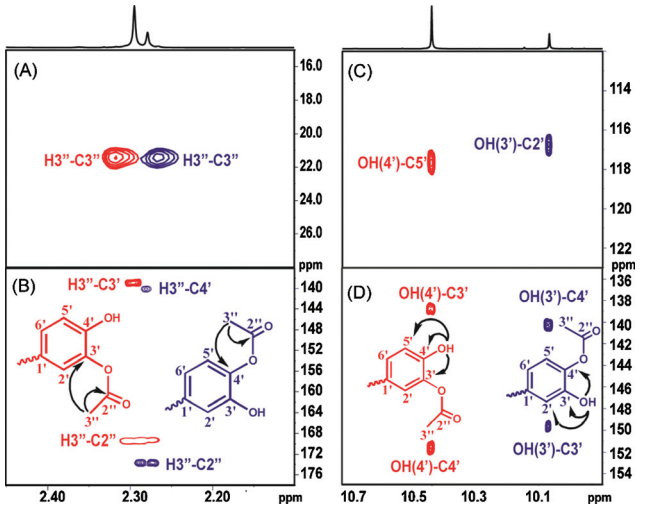

Fig. 3 Selected regions of the 500 MHz ^{1}H–^{13}C HSQC (A) and ^{1}H–^{13}C HMBC (B)–(D) spectra of the solution used in Fig. 2(C). The cross peaks corresponding to the 3′-and the 4′-monoacylated derivatives of quercetin are colored in red and blue, respectively. The cross peaks of the proton H3″ of the acetyl group to carbons C(2″) [$^{2}J(^{1}H,^{13}C)$] and C3′ [$^{4}J(^{1}H,^{13}C)$] for the 3′-derivative and the cross peaks of the proton H3″ of the acetyl group to carbons C(2″) [$^{2}J(^{1}H,^{13}C)$] and C4′ [$^{4}J(^{1}H,^{13}C)$] for the 4′-derivative are illustrated as arrows in the relevant chemical substructure parts in (B). The cross peaks of the proton OH(4′) to carbons C(5′) [$^{3}J(^{1}H,^{13}C)$] and C3′ [$^{3}J(^{1}H,^{13}C)$] and C4′ [$^{2}J(^{1}H,^{13}C)$] for the 3′-derivative and the cross peaks of the proton OH(3′) to carbons C(2′) [$^{3}J(^{1}H,^{13}C)$], C3′ [$^{2}J(^{1}H,^{13}C)$] and C4′ [$^{2}J(^{1}H,^{13}C)$] for the 4′-derivative are illustrated as arrows in the relevant chemical substructure parts in (C), (D).

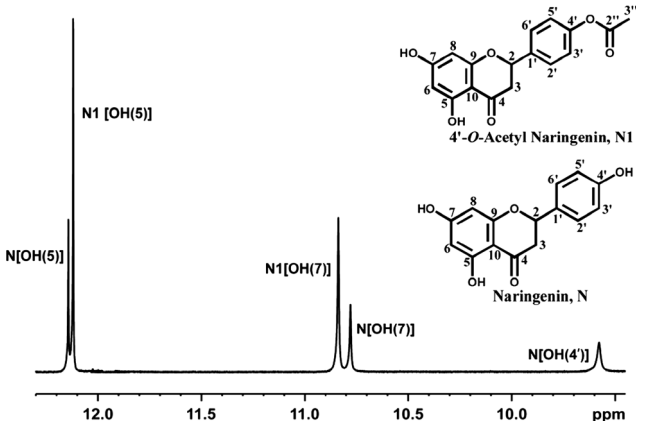

Fig. 4 OH region of the 500 MHz 1D ^{1}H NMR spectrum recorded of the crude reaction products of the enzymatically obtained monoacylated derivative of narigenin (2.30 μmol) in DMSO-d_6. Assignments of the resonances of the unreacted naringenin (N) and the monoacylated product (N1) are denoted.

the B ring, Scheme 1). The two acylated products did not present the same yield, but the 3′-*O*-acetyl derivative was formed with more than 3 times higher yield. We estimated that 1.012 μmol of 3′- and 0.337 μmol of the 4′-acylated product were present in the reaction products on the basis of the defined amount of picric acid which was added to the solution. This observed preference of the 3′ position of quercetin for CALB-mediated transformation to the relevant ester is in accordance with our docking calculations (Fig. 1).

From this work it became evident that the absorptions of the deshielded OH(5) protons of flavonoids (Fig. 2C) are not only deficient of resonance overlapping problems, but also could be used as an internal sensor for the rapid and direct monitoring of product formation in enzyme-mediated flavonoid aglycones transformations. Interestingly, this absorption, located in ring A, presents a great shielding sensitivity to substituent effects of at least up to eleven bonds apart (reaction took place in ring B). To further validate the usefulness of this approach, the enzyme-mediated naringenin aglycone (N, Fig. 4) transformation was investigated using the same optimum conditions as used for quercetin. The absence in naringenin of a double bond at position C2–C3 (Fig. 4) lead to increased flexibility and thus could result in higher yield, whereas the presence of a single phenolic group in the B ring (4′-position) could suggest the formation of single product. Indeed, as shown in Fig. 4, without any prior isolation of the reaction products, by just monitoring the OH(5) absorptions in the crude reaction mixture, we could clearly assign a single reaction product (N1, Fig. 4) having a yield of 71%. The same approach was also applied in the case of the enzymatic transformation of the glycosylated flavonoid hyperoside (3-*O*-galactoside of quercetin) using vinyl acetate. Again after addition of picric acid (the molar ratio of picric acid and acetylated derivative was 1 : 44) in the crude reaction products a high resolution 1D ^{1}H NMR spectrum was recorded (ESI, Fig. S16†). By simply using the OH(5) absorption, that was now located twelve bonds apart from the acylation site (sugar moiety), a single reaction product was determined with a 97% yield.

The determined efficiency of CALB to transform, in a regioselective way, flavonoid aglycones to their relevant esters could open a new avenue towards the production of more potent polyphenol derivatives. To this direction will also contribute our NMR methodology, which can rapidly screen product formation and achieve enhanced spectral resolution after the addition of picric acid. Transformation efficacy and regioselectivity could be readily monitored after directly recording the 1D ^{1}H NMR spectra of the crude reaction products without any prior isolation or fractionation. The absorption of the OH(5) proton of flavonoids aglycones which was used here as an internal sensor of the enzyme-mediated transformation competence could assist in the future *in situ* investigation of enzyme kinetics of such transformations.

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