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Combinatorial approach towards synthesis of 2′,3′-dideoxynucleosides and enzyme-catalysed selective hydrolysis of diethyl acetamidomalonate and amides of polyacetoxy aromatic carboxylic acid

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Seventeen novel 3′-alkylthio-2′,3′-dideoxynucleosides have been synthesised by Michael-type addition of alkylthiols to an α,β-unsaturated hexose aldehyde, followed by acetylation, nucleoside coupling and deprotection. Based on these results, a general scheme for combinatorial synthesis of libraries of 3′-substituted 2′,3′-dideoxynucleosides has been proposed. Porcine pancreatic lipase (PPL) has been found to hydrolyse the amides of polyacetoxyaromatic carboxylic acids in a highly chemoselective fashion. The enzyme exclusively hydrolyses the ester group over the amide group. Hydrolysis of diethyl acetamidomalonate in phosphate buffer in the presence of α-chymotrypsin proceeds enantioselectively affording the (+)-monoacid.

Search for reactions and processes that have greater selectivity and are more environment-friendly have resulted in the development of enzymes and microorganisms-catalysed reactions. Hydrolytic enzymes, particularly lipases have found widespread applications in manipulating protecting groups in organic synthesis because of low cost, wide-versatility, easy use and non- requirement of added cofactors[1]. We have earlier demonstrated the capabilities of lipases from porcine pancreas (PPL) and *Candida cylindracea* (CCL) in regioselective deacetylation of acetoxy group(s) other than the one at the *ortho* or *peri* position(s) to the nuclear (keto) carbonyl group in different classes of peracetylated polyphenolic compounds, *i.e.* aryl-alkyl ketones[2], chalcones[3], deoxybenzoins[4], flavonoids, coumarins and chromones[5]. On the basis of these results, we had proposed a general mechanism of deacetylation catalysed by PPL involving the formation of transient (dynamic) Schiff's base type complex by the nuclear carbonyl group present in the substrate with the free ζ-amino function of the lysine present in the active site of the lipase (analogy to the human pancreatic lipase)[2,6]. The formation of this complex results in embeddment of the *ortho* or the *peri* acetoxy function under the hydrophobic bulk of the enzyme and the nucleophilic serine -OH of the enzyme directs deacetylation of the other suitably placed acetoxy function(s) in the substrate molecule.

Polyhydroxyaromatic acid amides and their derivatives form an important class of natural compounds[7] and they play significant role in the biosynthesis of other polyphenolic natural products. The carbonyl group of amides is not known to form Schiff's base easily, and therefore may be used as chemical probe for confirmation of our above proposed mechanism of PPL action in enzymatic deacetylation. As per the proposed mechanism, it is expected that deacetylation of amides of polyacetoxy-aromatic acids catalysed by PPL would not involve formation of Schiff's base complex efficiently, and thus random placement of the different acetoxy groups of the substrate in the active site of the lipase would result in the formation of *ortho* hydroxy product together with other possible prod-

uct(s). With this aim, we have studied PPL-catalysed deacetylation reactions on eight diacetoxybenzamides **1-8** in dry tetrahydrofuran (THF) containing added *n*- butanol at 30°C; such biotransformation studies on the amides of polyacetoxyaromatic acids are not reported in the literature.

Aminomalonic acid (Ama, **26**) is the first representative of the homologous α-amino dicarboxylic acid series, including important proteinogenic acids, such as glutamic and aspartic. Ama has never been considered a proteinogenic amino acid until after it was detectd in the alkaline hydrolysates of *Escherichia* coli proteins[8]. Recently, peptide derivatives of Ama have invoked significant interest because of their possible physiological activities as enzyme inhibitors (of renin[9,10] and HIV-I proteases[11]).

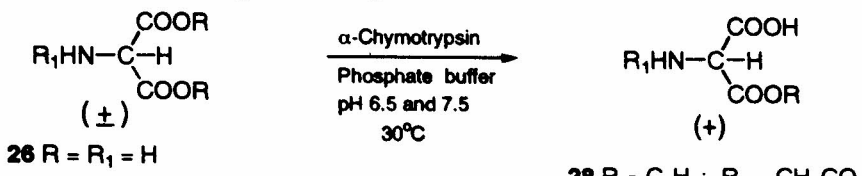
The Ama molecule has a prochiral center and all derivatives having different substituents at the two carboxyl groups would be chiral. Obviously, the chirality of Ama-derivatives might become an important stereocontrolling element if an Ama residue is to be incorporated in a peptide chain. Though there are reports on the separation of diastereoisomerically and enantiomerically pure *N*-Ama-PheOR derivatives of Ama[12], no enantiomerically enriched Ama derivatives, with only one asymmetric atom like in **28** have been obtained so far. Earlier attempts of enzymatic hydrolysis of AcNHCH $(COOEt)_2$ **27** with carboxyesterase NP[13] or α-chymotrypsin (α-CHT)[14] as catalysts afforded racemic AcNHCH(COOH)(COOEt) **28** as the only product. In the present work, we have reinvestigated the α-CHT- catalysed hydrolysis of **27**, and have found that the hydrolysis furnishes the (+) half ester **28** (Scheme I).

A number of 2′,3′-dideoxynucleoside analogs, *e.g.* 3′-azido-2′,3′-dideoxythymidine (AZT), 2′,3′- dideoxycytidine (ddC), 3′-azido-2′,3′-dideoxyuridine (AZDU) and 3′-azido-2′,3′-dideoxy-5-methylcytidine (AZddMeC) have been found to possess antiviral activity against HIV-I[15,16]. Indeed, the importance of this class of compounds is indicated by the fact that among the eight synthetic, clinically approved antiviral drugs, seven are nucleoside analogs[15]. Owing to this characteristic activity and worldwide need for an effective drug to combat AIDS, a large number of 3′-substituted 2′,3′- dideoxynucleoside analogs have been synthesised. In the course of our drug development programme, we have synthesised seventeen different stereoisomers of 3′-alkylthio- 2′,3′-dideoxy-nucleosides (**32a-c** and **33a-c**, Scheme II) and on the basis of these results, we have proposed a general scheme for combinatorial synthesis of libraries of 3′-substituted 2′,3′-dideoxynucleosides (Figure 1).

Results and Discussion

Deacetylation of amides of 2,4-diacetoxy- and 2,5-diacetxybenzoic acids

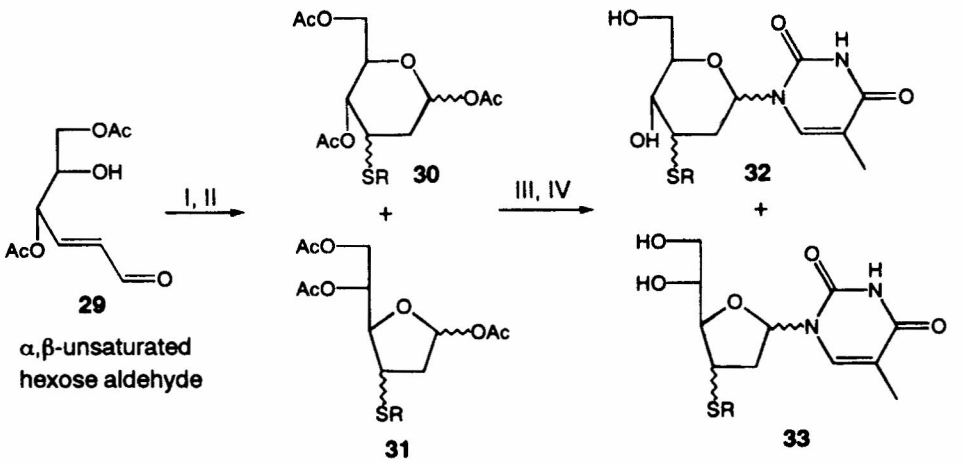
In the present investigation, we have studied the enzymatic deacetylation of eight different primary and secondary amides of 2,4- diacetoxy-and 2,5-diacetoxybenzoic acids, *i.e.* 2,4- diacetoxybenzamide **1**, 2,5-diacetoxybenzamide **2**, *N*-ethyl-2,4-diacetoxybenzamide **3**, *N*-ethyl-2,5-diacetoxybenzamide **4**, *N*-benzyl-2,4-diacetoxybenzamide **5**, *N*-benzyl-2,5- diacetoxybenzamide **6**, *N*-(4-methyl) phenyl- 2,4-diacetoxybenzamide **7** and *N*-(4-methyl) phenyl-2,5-diacetoxybenzamide **8** in dry THF in the presence of PPL. The results (Table I) show that deacetylation of both acetoxy groups takes place irrespective of their position, whereas the amide bond remains intact in all the eight compounds. Thus, the deacetylation of primary amides **1** and **2** catalysed by PPL afforded the dihydroxy compounds **10** and **12**, respectively as the major products alongwith the formation of 2-acetoxy-4-hydroxybenzamide **9** and 2-acetoxy-5-hydroxybenzamide **11**, respectively. *N*-Ethylbenzamides **3** and **4** and *N*-(4-methyl) phenyl-2,5-diacetoxybenzamide **8** on PPL mediated deacetylation afforded all the three possible products, *i.e.* 2-hydroxy-4/5-acetoxy-, 2-acetoxy-, 4/5- hydroxy- and dihydroxybenzamides **13**, **14**, **15**, **16**, **17**, **18**; and **23**, **24**, **25**, respectively. The deacetylation of *N*- benzylbenzamides **5** and **6** is highly selective and the enzyme exclusively cleaves the *ortho* acetoxy function leading to the formation of compounds **19** and **20** in 50% and 65% yields, respectively.



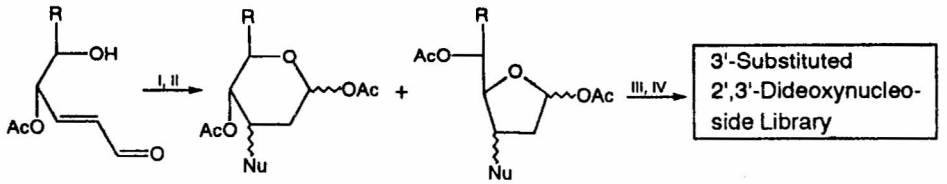
26 R = R_1 = H
27 R = C_2H_5; R_1 = CH_3CO

28 R = C_2H_5; R_1 = CH_3CO

Scheme I



Scheme II



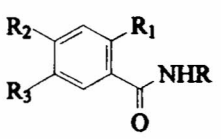
Example: 5 Aldehydes x 5 nucleophiles x 5 nucleobases x 8 stereoisomers =1000 member-library

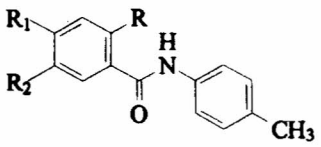
Figure 1

In all these cases, except with the primary amides **1** and **2**, deacetylation of the *ortho* acetoxy function is favoured. This is illustrated clearly in the case of *N*-(4-methyl) phenyl-2,4-diacetoxybenzamide **7** which afforded 2-hydroxy-4-acetoxybenzamide **21** as the major product (in 70% yield). These results of enzymatic deacetylation carried out on eight benzamides revealed that PPL does not show exclusive preference for one out of two chemically different acetoxy functions though the predominant products formed are the thermodynamically stable *ortho* hydroxy compounds due to intramolecular hydrogen bonding between the amide carbonyl moiety and the ortho hydroxy function. This is reverse to results obtained by us during PPL- catalysed deacetylation of poldyacetoxy aromatic ketones, where due to Schiff's base complex formation between the keto carbonyl group and ζ-amino function of the lysine, the enzymatic deacetylation does not occur at the *ortho* acetoxy function[2,6]. The amido carbonyl moiety cannot participate in Schiff's base formation, wherefore PPL deacetylates the *ortho* acetoxy function in amides which is evidenced in the present work. Therefore our earlier proposed Schiff's base model mechanism of PPL-catalysed deacetylation of polyacetoxy aromatic ketones in organic solvents is supported[2,6].

Enantioselective hydrolysis of diethyl acetamidomalonate catalysed by α-chymotrypsin

The hydrolysis of AcNHCH $(COOEt)_2$ **27** catalysed by α- CHT proceeded enantioselectively with the formation of monoester (+) **-28** which gradually racemised during the reaction. Reactions were carried out at ambient temperature in 0.01 *M* phosphate buffer solutions in the presence of α-CHT (40- 60 units/mg of protein). The progress of the reactions were monitored by titration as the *p*H was maintained





1 R = R_3 = H, R_1 = R_2 = $OCOCH_3$
2 R = R_2 = H, R_1 = R_3 = $OCOCH_3$
3 R = C_2H_5, R_1 = R_2 = $OCOCH_3$, R_3 = H
4 R = C_2H_5, R_1 = R_3 = $OCOCH_3$, R_2 = H
5 R = $CH_2C_6H_5$, R_1 = R_2 = $OCOCH_3$, R_3 = H
6 R = $CH_2C_6H_5$, R_1 = R_3 = $OCOCH_3$, R_2 = H
9 R = R_3 = H, R_1 = $OCOCH_3$, R_2 = OH
10 R = R_3 = H, R_1 = R_2 = OH
11 R = R_2 = H, R_1 = $OCOCH_3$, R_3 = OH
12 R = R_2 = H, R_1 = R_3 = OH
13 R = C_2H_5, R_1 = OH, R_2 = $OCOCH_3$, R_3 = H
14 R = C_2H_5, R_1 = $OCOCH_3$, R_2 = OH, R_3 = H
15 R = C_2H_5, R_1 = R_2 = OH, R_3 = H
16 R = C_2H_5, R_1 = OH, R_2 = H, R_3 = $OCOCH_3$
17 R = C_2H_5, R_1 = $OCOCH_3$, R_2 = H, R_3 = OH
18 R = C_2H_5, R_1 = R_3 = OH, R_2 = H,
19 R = $CH_2C_6H_5$, R_1 = OH, R_2 = $OCOCH_3$, R_3 = H
20 R = $CH_2C_6H_5$, R_1 = OH, R_2 = H, R_3 = $OCOCH_3$

7 R = R_1 = $OCOCH_3$, R_2 = H
8 R = R_2 = $OCOCH_3$, R_1 = H
21 R = OH, R_1 = $OCOCH_3$, R_2 = H
22 R = R_1 = OH, R_2 = H
23 R = OH, R_1 = H, R_2 = $OCOCH_3$
24 R = $OCOCH_3$, R_1 = H, R_2 = OH
25 R = R_2 = OH, R_1 = H

Table I — Deacetylation studies on 2,4-diacetoxy- and 2,5- diacetoxybenzamides catalysed by PPL in dry THF containing 1-2% *n*-butanol at 30°C

Substrate	Reaction time (days)	Product(s) (% yield)
2,4-Diacetoxybenzamide **1**	5	2-Acetoxy-4-hydroxybenzamide **9** (30%) and 2,4-dihydroxybenzamide **10** (40%)
2,5-Diacetoxybenzamide **2**	5	2-Acetoxy-5-hydroxybenzamide **11** (20%) and 2,5-dihydroxybenzamide **12** (60%)
N-Ethyl-2,4-diacetoxybenzamide **3**	5	*N*-Ethyl-2-hydroxy-4-acetoxybenzamide **13** (35%), *N*-ethyl-2-acetoxy-4- hydroxybenzamide **14** (30%) and *N*-ethyl-2,4-dihydroxybenzamide **15** (15%)
N-Ethyl-2,5-diacetoxybenzamide **4**	5	*N*-Ethyl-2-hydroxy-5-acetoxybenzamide **16** (40%) *N*-ethyl-2-acetoxy-5-hydroxybenzamide (**17**) (15%) and *N*-ethyl-2,5-dihydroxybenzamide (**18**) (5%)
N-Benzyl-2,4-diacetoxybenzamide **5**	5	*N*-Benzyl-2- hydroxy-4-acetoxybenzamide **19** (50%)
N-Benzyl-2,5-diacetoxybenzamide **6**	5	*N*-Benzyl-2- hydroxy-5-acetoxybenzamide **20** (65%)
N-(4-Methyl)phenyl-2,4-diacetoxybenzamide **7**	10	*N*-(*4*-Methyl)phenyl-2-hydroxy-4-acetoxybenzamide **21** (70%) and *N*-(4-methyl) phenyl-2,4-dihydroxybenzamide **22** (5%)
N-(4-Methyl)phenyl-2,5-diacetoxybenzamide **8**	10	*N*-(4-Methyl)phenyl-2-hydroxy-5-acetoxybenzamide **23** (65%)
		N-(4-methyl) phenyl-2-acetoxy-5-hydroxybenzamide **24**(15%) and *N*-(4-methyl) phenyl-2,5-dihydroxy-benzamide **25** (10%).

* No deacetylation reaction was observed on incubation of the substrates under indentical conditions without adding PPL.

either at 6.5 or 7.5 by addition of 1*M* NaOH solution. Only one equivalent of NaOH was needed indicating that only one of the two ester groups of **27** was hydrolysed to afford **28**. The results indicated that reaction proceeds faster at *p*H 7.5 than at 6.5 in accordance with the *p*H-activity profile of α-CHT[17].

The hydrolysis was accompanied initially by an increase of the positive optical rotation (α-obs) of the solution at 578 nm. Taking into account that the contribution of α-CHT to the observed rotation is negligible, the optical activity should only be associated with the formation of (+)-**28**. It may be mentioned that the angle of rotation of α-CHT at *p*H 7.5 is -0.021 and that at *p*H 6.5 is -0.027, the optical rotation of our reaction mixture was in the range +1.01 to +1.09 at *p*H 7.5 and in the range + 0.31 to + 0.76 at *p*H 6.5[18]. The specific rotation of (+)-**28** at different stages of the reaction could be easily calculated from the data and the extent of the substrate conversion. The time dependence of α-obs was not linear and at high conversion of **27**, a decrease of α-obs was observed. At the same time, the specific rotation, [α] continuously decreased during the course of reaction. These two facts indicate that the product (+)-**28** formed gets racemised in the reaction giving rise to (±)-**28**. Owing to the racemisation reaction, we were unable to determine the enantioselectivity of α- CHT-catalysed hydrolysis of **27** and absolute configuration of (+)-**28**. Nevertheless, the latter can be deduced, assuming that stereochemical orientation of AcNH and H moieties of the α-C of **27** in the active site of α-CHT was the same as for its specific substrates, i.e. the aromatic *N*-acetyl-L-amino acid esters[18]. In this case, pro-*S* COOEt group would be placed in the hydrophobic pocket of the enzyme active site and pro-*R* COOEt group would be subjected to the attack of the nucleophilic OH group of the enzyme, thus affording products with *(R)*-configuration. The extrapolation of the value of $[\alpha]_{578}$ to the beginning of the hydrolysis reaction gave +61 as the value for the specific rotation of the initially formed unracemized sample of *(R)*-**28**. Work to estimate the real enantiomeric excess *(e.e.)* of the reaction is underway.

Combinatorial synthesis of 3'-alkylthio-2',3'-dideoxynucleosides

Michael-type addition of ethanethiol, followed by acetylation and flash column chromatography on a short silica gel column afforded a mixture of three 2,3-dideoxy-3-ethylthiopyranoses **30a** and two 2,3-dideoxy-3-ethythiofuranoses **31a** in 74% yield (**30a:31a**~5:1). The formation of products with furanose configuration implies a 4-O to 5-O accetyl migration before acetylation of the anomeric position as observed earlier using other nucleophiles.[19] Addition of ethanethiol to aldehyde **29** employing slightly different reaction conditions has earlier been reported[20] to give exclusively pyranose products in 82% yield without acylation. Following same procedure as for **30a/31a**, addition of 1-propanethiol and 1-butanethiol and subsequent acetylation afforded a mixture of three pyranoses **30b** and two furanoses **31b** (80% yield; **30b:30b**~2:1), and a mixture of three pyranoses **30c** and four furanoses **31c** (86% yield; **30c:31**~3:1), respectively. From these experiments, it seems that the longer the alkyl chain, the more furanose product is being formed. Conversion of each of the three mixture **30/31** into 3'-alkylthio 2', 3'-dideoxynucleoside derivatives was achieved using standard conditions[21-23]. (Scheme II); after deprotection and flash column chromatography on a short silica gel column, a mixture of three pyranosyl nucleosides **32a** and two furanosyl nucleosides **33a** (88% yield; **32a:33a**, ~5:1), a mixture of three pyranosyl nucleosides **32b** and two furanosyl nucleosides **33b** 89% yield; **32b:32b,**~5:2) and a mixture of three pyranosyl nucleosides **32c** and four furanosyl nucleosides **33c** (90% yield; **32c:33c**, ~1:1) was obtained.[24]

Thus, using this strategy, three small sub-libraries of five, five and seven nucleosides have been synthesised.[24] Based on these results, we propose a general scheme for synthesis of 3'-substituted 2',3'-dideoxynucleosides in a combinatorial fashion (Figure 1). As an example, reaction of five different α-β-unsaturated carbohydrate aldehydes with five different nucleophiles, followed by nucleoside coupling with five different nucleobases should afford 125 different compounds. However, if the formation of two 3-epimeric pyranoses and two 3-epimeric furanoses, affording four carbohydrate intermediates, and the formation of two anomeric nucleosides for each intermediate is a consequence of the chemistry of this strategy, a significant increase in the diversity of the library should result leading to the formation of a library of a maximum of 1000 compounds.

The present study has shown interesting and potentially useful chemoselectivity during PPL-catalysed deacetylation reactions in dry THF on polyacetoxyaromatic carboxylic acid amides. The

lipase performs exclusive desterification of the ester groups over the amide functionality in all the eight substrates under study. In most of the cases, the lipase selectively hydrolyses the *ortho* acetoxy group of benzamides in as high yield as 70% which is difficult to achieve by purely chemical methods. This study also supports our earlier proposed hypothesis of Schiff's base formation during deacetylation reactions on polyacetoxyaromatic ketones catalysed by PPL in dry organic solvents. In view of the physiological importance of enzyme inhibitors, aminomalonic acid has stimulated much interest in the synthesis of its optically active monoester form which can be incorporated in peptide chains as a marker and stereocontrolling element. As a first step, we have demonstrated the enantioselective hydrolysis of diethyl acetamidomalonate and are presently searching for suitable conditions to control the inherent racemisation. Synthesis of 3′-alkylthio-2′,3′-dideoxynucleosides, analogs of AZT, and our proposition of the strategy for the synthesis of large libraries of nucleosides in a combinatorial manner, may find utility in pharmaceutical research.

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