POTENTIAL PRODRUGS OF 6-ACETYLMETHYLENEPENICILLANIC ACID (Ro 15-1903)

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The synthesis and biological activities of a series of non-classical penicillins are described. These compounds were synthesized by treating the pivaloyloxymethyl ester of 6-acetylmethylenepenicillanic acid (Ro 15-1903) with various nucleophiles. They were found to be less active against the β-lactamases from *Proteus vulgaris* 1028, *Escherichia coli* 1024, *Klebsiella pneumoniae* NCTC 418 and *E. coli* RTEM than the parent compound. Nevertheless, synergy with ampicillin against whole bacterial cells producing β-lactamases was evident, although the single compounds did not exhibit antibacterial properties. With the compounds **2a** and **2b**, synergistic interaction with ampicillin could also be demonstrated in mice.

In previous papers we reported on the biological properties of a new class of potent β-lactamase inhibitors[1~4]. Application of these inhibitors *in vivo*, especially for therapeutic purposes, is limited because they are unstable in biological fluids. Their highly reactive functional groups react with other nucleophilic medium components in a non-specific way. This instability and high reactivity is responsible for the discrepancy observed between the enzymatic data and the *in vitro* and *in vivo* results[5].

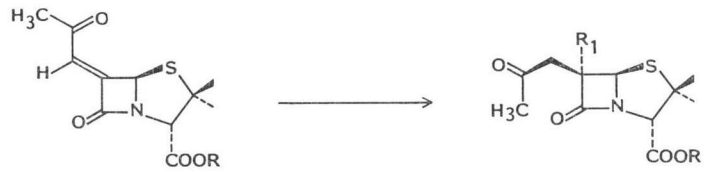
The investigations reported here had two main objectives. The first was to design a prodrug of these β-lactamase inhibitors in an attempt to obtain a greater (chemical) stability but retain site specific delivery. The second was to find out new structures with potential β-lactamase activity.

Chemistry

The high reactivity of the two functionalities present in the acylmethylenes, *viz.* the ketone and the MICHAEL acceptor was exploited (Schemes 1 and 2). We first tried the base catalyzed addition of sulfur compounds. Reaction of **1b** with one equivalent of thiophenol in the presence of catalytic amounts of 1,5-diazabicyclo(3,4,0)nonene-5 (DBN) at 20°C gave a clean reaction and compound **2a** was isolated. The addition was expected to occur from the α face although in these compounds the steric hindrance of the β face is not as important as in the case of classical penicillins[6]. The evidence for assigning the thioether substituent to the 6-α-position came from nuclear Overhauser effect (NOE).

Similarly, reaction of **1b** with thioacetic acid gave the expected MICHAEL reaction product **2b** in 58% yield and the side product **4**. The formation of **4** involves addition of thioacetic at C8 with a concomitant cleavage of the S1-C5 bond, followed by intramolecular attack of the thiolate at the β-lactam. The compound was obtained in 11% yield as a 1 : 1 mixture of diastereomers. On reaction with one equivalent of 2-mercaptopyridine an unexpected fragmentation occurred, leading to the thiazoline **5** in 76% yield. The corresponding carboxylic acid has also been isolated from the reaction of benzylpenicillin with D-alanine carboxypeptidase[7] as well as after treatment of benzylpenicillin with trifluoroacetic acid[8]. In our case the fate of the acylmethylene portion of **1b** remained uncertain because the isolated yield of **6** (1%) after chromatography did not corresponded to the isolated yield of **5**.

Scheme 1.



1b **2a ~ d**

(**1a** R = H)
1b R = $CH_2OCOC(CH_3)_3$

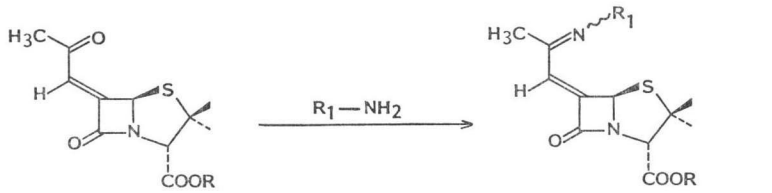
2a R_1 = —S— 

2b $R_1 = SCOCH_3$

2c R_1 = —N 

2d $R_1 = NHNHCOOC_2H_5$

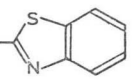
Scheme 2.



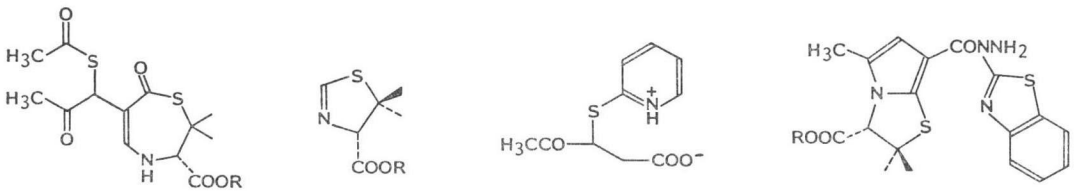
1b **3a ~ d**

3a $R_1 = OH$

3b $R_1 = OCH_3$

3c R_1 = —NH— 

3d $R_1 = NHCOOC_2H_5$



4 **5** **6** **7**

The reaction of **1b** with nitrogen nucleophiles was more complex. When **1b** reacted with one equivalent of morpholine in methylene chloride at 20°C the single crystalline compound **2c** was isolated in 83% yield. In this case too the addition occured at C6 from the α face. The stereochemistry was confirmed once more by NOE. In contrast, however, hydroxylamine (1 equivalent in ethanol at 20°C) preferentially attacked the ketone and not the β-lactam. This was surprising since it is well established

Table 1. Inhibition of isolated β-lactamases, the assay carried out in the absence (−E) or presence (+E) of hog liver esterase.

Compound	IC_{50} (μM)							
	P. vulgaris 1028		*K. pneumoniae* NCTC 418		*E. coli* 1024		*E. coli* RTEM	
	−E	+E	−E	+E	−E	+E	−E	+E
1a	0.004	—	0.005	—	1.7	—	0.007	—
1b	0.16	0.015	0.19	0.001	32	1.5	0.02	ND
2a	3.7	0.3	44	0.1	>100	7.5	0.8	0.002
2b	4.2	0.2	4.6	0.1	60	14	0.7	0.01
2c	4.1	0.1	6.3	0.1	>100	10	0.9	0.01
2d	1.7	1.7	16	2	100	100	100	0.048
3a	0.46	0.035	0.71	0.05	100	2.7	0.06	0.002
3b	>100	3.4	>100	6.1	>100	1.4	>100	6.1
3c	>100	1.5	>100	1.6	15	7.5	100	0.1
Clavulanic acid	0.03	—	0.02	—	85	—	0.04	—

ND: Not done.

that the lactam amide bond in penicillins is very susceptible to cleavage by nucleophiles[9]. The oxime **3a** was obtained in 82% yield as a mixture of geometrical isomers which could not be separated by flash chromatography. The reaction with methoxylamine gave the methoximes **3b** in 80% yield as a mixture of geometrical isomers. It was then expected that **1b** would afford the carbazone when reacted in ethanol with ethyl carbazate. Indeed, when subjected to the above conditions, a complex mixture was obtained from which two compounds **2d** and **3d** could be isolated by flash chromatography in low yield (11% and 18% respectively); compound **2d** resulted from MICHAEL addition of ethyl carbazate at C6. The reaction of **1b** with 2-hydrazinobenzothiazole was also complex. When **1b** was reacted with one equivalent of the hydrazine, the hydrazone **3c** was obtained in 38% yield. The side product **7** was also isolated in 12% yield. This compound presumably arose from the opening of the β-lactam, followed by a rapid intramolecular rearrangement. This type of rearrangement has been described previously[4].

Biological Results

Inhibition of β-Lactamases

Table 1 shows the inhibitory properties of the compounds. They were evaluated as described[2]. Hog liver esterase was used to cleave esters during the preincubation period. The modifications on the side chain of **1b** dramatically reduced the β-lactamase-inhibitory activity. The oxime **3a** is the most active compound of the series. Against the β-lactamase from *Proteus vulgaris* 1028 and *Klebsiella pneumoniae* NCTC 418, **3a** (after ester-cleavage) was found to be nearly as active as clavulanic acid. Against the β-lactamase from *Escherichia coli* 1024 and *E. coli* RTEM, **3a** is much more active than clavulanic acid.

Antibacterial Activity and Synergy

The antibacterial properties of the compounds, alone and in combination with ampicillin, were investigated *in vitro* (Table 2) and *in vivo* (Table 3) using the methods previously described[3]. The compounds alone exhibited no antibacterial properties. However, when combined with ampicillin, there was a decrease in the MIC of ampicillin against *K. pneumoniae* NCTC 418, *E. coli* 1024, *P. vulgaris* 1028 and particularly *Staphylococcus aureus* 887. The synergy was most evident with compounds **2a**, **2b** and **2c**. In combination with ampicillin (ratio, 1 : 1), **2b** was approximately equivalent to **1b** in pro-

Table 2. *In vitro* activity in combination with ampicillin*.

Compound	Compound**+ampicillin** (MIC, μg/ml)			
	P. vulgaris 1028	*K. pneumoniae* NCTC 418	*E. coli* 1024	*S. aureus* 887
1b	3.1+3.1	6.3+6.3	50+50	0.8+0.8
2a	12.5+12.5	25+25	50+50	1.6+1.6
2b	6.3+6.3	12.5+12.5	50+50	0.8+0.8
2c	6.3+6.3	12.5+12.5	50+50	3.1+3.1
3a	12.5+12.5	50+50	50+50	3.1+3.1
3b	>50+50	>50+50	>50+50	12.5+12.5
3c	>50+50	>50+50	>50+50	25+25
Clavulanic acid	1.6+1.6	1.6+1.6	50+50	0.8+0.8

* Inoculum, 10^5 cfu/ml.
** MIC single compounds, >50 μg/ml.

Table 3. *In vivo* activity in combination with ampicillin.

Compound	ED_{50} (mg/kg, sc) compound*+ampicillin*		
	P. vulgaris 1028	*K. pneumoniae* NCTC 418	*S. aureus* 887
1b	21+21	40+40	9+9
2a	25+25	50+50	>50+50
2b	12+12	>50+50	14+14
2c	>50+50	>50+50	>50+50
2d	>25+25	>50+50	>25+25
3a	>25+25	>50+50	>50+50
3b	>25+25	>50+50	>50+50
3c	>25+25	>50+50	>50+50

* ED_{50} single compounds, >50 mg/kg, sc.

tecting mice against systemic infections with *P. vulgaris* 1028 and *S. aureus* 887 after subcutaneous administration. Except for **2a**, which showed some efficacy against *P. vulgaris* 1028 and *K. pneumoniae* NCTC 418, when combined with ampicillin, the other compounds were inactive in mice.

Discussion

As can be seen, there are no good correlations between the enzyme inhibition data (Table 1) and the antibacterial synergy results (Table 2), particularly for compounds **2a**, **2b**, **2c** and **3a**. Different stabilities could possibly explain these discrepancies; *e.g.* **3a** could be less stable in the test medium than **2a**, **2b** and **2c**. It can be assumed that differences in the ease of penetration of these compounds through the outer membrane of the bacteria also play a role. Furthermore, **2a**, **2b** and **2c** could be assumed to act either unchanged or after metabolic transformation into **1b**. There are no adequate answers yet available to distinguish between these possibilities. The *in vivo* data show that one aim, to design a prodrug of **1a** with improved activity has only been partially achieved.

Experimental

The methods were the same as described[6].

Methylene (2*S*,5*R*)-6-[2-(Hydroxyimino)propylidene]penicillanate Pivalate (**3a**)

A solution of 0.139 g (2 mmol) of hydroxylamine hydrochloride in 10 ml EtOH was treated under stirring with 0.164 g (2 mmol) of sodium acetate at 20°C. To this mixture a solution of 0.73 g (2 mmol) methylene (6*Z*)-6-acetonylidenepenicillanate pivalate in 30 ml EtOH was then added. After 1.5 hours the solvent was evaporated and the residue was partitioned between Et_2O and H_2O. The organic phase

was separated, washed with H_2O, dried ($MgSO_4$), filtered and evaporated. The residue was purified by flash chromatography yielding 0.63 g of a mixture of geometrical isomers. Eluent cyclohexane - EtOAc (7 : 3): IR ($CHCl_3$) cm^{-1} 3580, 3350, 1770, 1604; MS *m/z* 384, 367, 337, 280, 274; ^1H NMR (60 MHz, $CDCl_3$, data of the main component) δ 1.25 (s, $(CH_3)_3C$), 1.52 and 1.62 (2×s, 2-CCH_3), 2.06 (s, $CH_3C=N$), 4.59 (3-CH), 5.8 (d), 5.89 (d, J_{gem}=6 Hz, OCH_2O), 5.97 (d, J_{58}=1 Hz, 5-CH), 6.67 (d, J_{58}=1 Hz, 8-CH).

Methylene (2*S*,5*R*)-6-[(*E*/*Z*)-2-(Methoxyimino)propylidene]penicillanate Pivalate (**3b**)

A solution of 0.835 g (10 mmol) of methoxylamine hydrochloride in 100 ml EtOH was treated under stirring with 0.82 g (10 mmol) of sodium acetate at 20°C. To this suspension a solution of 3.7 g (10 mmol) methylene (6*Z*)-6-acetonylidenepenicillanate pivalate in 200 ml EtOH was added. After 4 hours the solvent was evaporated and the product was purified by flash chromatography yielding 3.2 g of a mixture of geometrical isomers. Eluent cyclohexane - EtOAc (8 : 2): IR ($CHCl_3$) cm^{-1} 1767, 1579, 1051; MS *m/z* 398, 367, 337, 294; ^1H NMR (60 MHz, $CDCl_3$, data of the main component) δ 1.25 (s, $(CH_3)_3C$), 1.52 and 1.6 (2×s, 2-CCH_3), 1.98 (s, $CH_3C=N$), 4.0 (s, CH_3O), 4.57 (s, 3-CH), 5.81 (d), 5.87 (d, J_{gem}=5 Hz, OCH_2O), 5.96 (singlet broadened by long range coupling, 5-CH), 6.58 (d, J_{58}=1 Hz).

Methylene (2*S*,5*R*,6*S*)-6-Acetonyl-6-morpholinopenicillanate Pivalate (**2c**)

A stirred solution of 1.845 g (5 mmol) of methylene (6*Z*)-6-acetonylidenepenicillanate pivalate in 40 ml of methylene chloride was treated dropwise with 0.435 ml (5 mmol) of morpholine at 20°C. After 22 hours the solvent was evaporated and the residue was purified by flash chromatography using cyclohexane - EtOAc (6 : 4) as eluent. Yield, 1.9 g colorless crystals: IR (KBr) cm^{-1} 2828, 1777, 1750, 1706, 1115; MS *m/z* 457 (M−1), 438, 369, 255; ^1H NMR (80 MHz, $CDCl_3$) δ 1.23 (s, $(CH_3)_3C$), 1.49 and 1.6 (2×s, 2-CCH_3), 2.29 (s, CH_3CO), 2.56 and 2.8 (m, CH_2N), 2.95 and 3.08 (AB-spectrum, J_{gem}= 17 Hz, CH_2CO), 3.66 (t, J=5 Hz, OCH_2), 4.44 (s, 3-CH), 5.48 (s, 5-CH), 5.75 (d), 5.88 (d, J_{gem}=5 Hz, OCH_2O).

Methylene (2*S*,5*R*,6*S*)-6-Acetonyl-6-(phenylthio)penicillanate Pivalate (**2a**)

To 1.84 g (5 mmol) of methylene (6*Z*)-6-acetonylidenepenicillanate pivalate dissolved in 30 ml of methylene chloride was added 0.51 ml (5 mmol) of thiophenol at 20°C. The mixture was stirred while a drop of DBN was added and the temperature rose to 25°C. The color of the mixture turned dark orange. After 10 minutes the solvent was removed under reduced pressure. The oil obtained was purified by flash chromatography. Eluent cyclohexane - EtOAc (8 : 2). Yield, 1.8 g of orange oil: IR ($CDCl_3$) cm^{-1} 1741, 1704; MS *m/z* 479, 369, 339, 274; ^1H NMR (80 MHz, $CDCl_3$) δ 1.22 (s, $(CH_3)_3C$), 1.41 and 1.55 (2×s, 2-CCH_3), 2.16 (s, $COCH_3$), 3.05 (d), 3.26 (d, J_{gem}=18 Hz, 8-CH_2), 4.34 (s, 3-CH), 5.5 (s, 5-CH), 5.71 (s, OCH_2O), 7.3～7.8 (m, Ar).

Methylene (2*S*,5*R*,6*R*)-6-Acetonyl-6-(acetylthio)penicillanate Pivalate (**2b**)

A solution of 1.845 g (5 mmol) of methylene (6*Z*)-6-acetonylidenepenicillanate pivalate in 25 ml methylene chloride was treated under stirring with 0.35 ml (5 mmol) of thioacetic acid at 20°C. A drop of DBN was added to the mixture. The temperature rose to 25°C. After 6 hours the crude product, obtained after evaporation of the solvent, was purified by flash chromatography to give two compounds. Eluent cyclohexane - EtOAc (7 : 3).

The first eluted material (1.3 g) was **2b**: IR ($CHCl_3$) cm^{-1} 1781, 1770, 1720; MS *m/z* 445, 412, 402, 385, 286, 256; ^1H NMR (80 MHz, $CDCl_3$) δ 1.21 (s, $(CH_3)_3C$), 1.47 and 1.60 (2×s, 2-CCH_3), 2.18 and 2.31 (2×s, $COCH_3$ and $SCOCH_3$), 3.3 (d), 3.5 (d, J=18 Hz, 8-CH_2), 4.46 (s, 3-CH), 5.44 (s, 5-CH), 5.74 (d), 5.84 (d, J=5 Hz, OCH_2O).

The second eluted material (0.13 g) was **4**: IR (KBr) cm^{-1} 3364, 1768, 1747, 1712, 1692, 1621; MS *m/z* 446 $(M+H)^+$, 372, 370, 298, 274, 240; ^1H NMR (80 MHz, DMSO-d_6, 1 : 1 mixture of two diastereomers) δ 1.16 (s, $(CH_3)_3C$), 1.46 and 1.52 (2×s, 2-CCH_3 of I and II), 2.08 (s, $COCH_3$), 2.28 and 2.31 (2×s, $SCOCH_3$ of I and II), 4.51 and 4.59 (2×d, J=2 Hz, CHCOO of I and II), 4.98 and 5.16 (2×s, SCH of I and II), 5.8 (center of two AB-spectra of OCH_2 of I and II, J_{gem}=5 Hz), 7.10 and 7.35 (2×d, J=8.5 Hz, CH= of I and II), 8.42 and 8.53 (2×br d, J=8.5 Hz, NH of I and II).

Methylene (2*S*,5*R*)-6-[[2-(2-Benzothiazolyl)hydrazono]propylidene]penicillanate Pivalate (**3c**) and Methylene (3*S*)-7-[2-(2-Benzothiazolyl)carbazoyl]-2,3-dihydro-2,2,5-trimethylpyrrolo[2,1-b]thiazole-3-carboxylate Pivalate (**7**)

2-Hydrazinobenzothiazole (1.65 g, 10 mmol) was stirred as a suspension in EtOH (75 ml) at 20°C, methylene (6*Z*)-6-acetonylidenepenicillanate pivalate (3.7 g, 10 mmol) in a mixture of EtOH (100 ml) and THF (30 ml) was added dropwise. A clear orange solution was obtained after 10 minutes. After 1 hour a precipitate appeared. The resulting suspension was concentrated to a volume of 50 ml. The yellowish crystals were filtered off to give 0.6 g of **7**: IR (KBr) cm^{-1} 1756, 1732, 1620; MS *m/z* 516, 501, 352; ^1H NMR (80 MHz, $CDCl_3$) δ 1.21 (s, $(CH_3)_3C$), 1.62 and 1.76 (2×s, 2-CCH_3), 2.13 (d, J=1 Hz, CH_3C=), 4.52 (s, CHCOO), 4.93 (s, NH_2), 5.8 (d), 5.9 (d, J_{gem}=5.5 Hz), 6.96 (m, J=1 Hz, HC=), 7.15~7.9 (m, Ar).

The filtrate was concentrated under reduced pressure. The residue was purified by flash chromatography using cyclohexane - EtOAc (7 : 3). Yield, 2 g of **3c** as orange foam: IR (KBr) cm^{-1} 3270, 1760, 1604, 1553, 1271; MS *m/z* 516, 501, 486; ^1H NMR (80 MHz, $CDCl_3$) δ 1.23 (s, $(CH_3)_3C$), 1.52 and 1.60 (2×s, 2-CCH_3), 2.08 (s, CH_3C=N), 4.61 (s, 3-CH), 5.8 (d), 5.9 (J_{gem}=5.5 Hz, OCH_2O), 6.07 (d, J=1 Hz, 5-CH), 6.64 (d, J_{58}=1 Hz), 7.05~7.8 (m, Ar), 9.05 (br s, NH).

Methylene (2*S*,5*R*)-6-[2-[2-(Ethoxycarbonyl)hydrazono]propylidene]penicillanate Pivalate (**3d**) and Methylene (2*S*,5*R*,6*S*)-6-Acetonyl-6-[2-(ethoxycarbonyl)hydrazino]penicillanate Pivalate (**2d**)

A stirred solution of 0.6 g (5.7 mmol) ethyl carbazate in 35 ml EtOH was treated dropwise at 20°C with 2.1 g (5.7 mmol) methylene (6*Z*)-acetonylidenepenicillanate pivalate in 50 ml EtOH. After 48 hours the solvent was evaporated and the residue was purified by flash chromatography.

Elution with cyclohexane - EtOAc (6 : 4) gave 0.3 g of yellow foamy **3d**: IR (KBr) cm^{-1} 3290, 1761, 1576, 1519, 1212; MS *m/z* 455, 341, ^1H NMR (80 MHz, $CDCl_3$) δ 1.23 (s, $(CH_3)_3C$), 1.35 (t, CH_3CH_2), 1.50 and 1.58 (2×s, 2-CCH_3), 1.98 (s, CH_3C=N), 4.33 (q, J=7 Hz, CH_3CH_2), 4.59 (s, 3-CH), 5.8 (d), 5.9 (d, J=5.5 Hz, OCH_2O), 6.03 (d, J_{58}=1 Hz, 5-CH), 6.69 (d, J_{58}=1 Hz, 8-CH), 8.25 (br s, NH).

0.5 g of **2d** were also isolated: IR (KBr) cm^{-1} 1763, 1716, 1263, 1112; ^1H NMR (80 MHz, $CDCl_3$) δ 1.22 (s, $(CH_3)_3C$), 1.27 (t, J=7 Hz, CH_3CH_2), 1.47 and 1.58 (2×s, 2×CCH_3), 2.22 (s, CH_3CO), 3.10 (s, 8-CH_2), 4.2 (q, CH_3CH_2), 4.45 (s, 3-CH), 5.61 (s, 5-CH), 5.8 (d), 5.86 (d, J=5.5 Hz, OCH_2O), 6.36 (br s, CONH), second NH not detected, assumed to be very broad on account of exchange with H_2O from solvent.

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