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Degradation Studies of Antibiotic MDL 62,879 (GE2270A) and Revision of the Structure

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Abstract: Fragments of the thiopeptide antibiotic MDL 62,879 (GE2270A) were prepared under mild degradation conditions. Their structures were determined by spectroscopic techniques and by comparison to synthetic reference thiazolyl dipeptides. The two thiazolyl amino acids have stereocenters with S configurations and their sequence is reversed with respect to the structure previously proposed for MDL 62,879.

MDL 62,879 (GE2270A) **1** is a new antibiotic produced by *Planobispora rosea* ATCC 53773[1]. It inhibits Gram-positive bacteria and anaerobes by acting on the protein synthesis Elongation Factor[2,3]. MDL 62,879 is a highly modified peptide, which belongs to the thiazolyl peptide group of antibiotics[4]. The previously proposed structure (Fig. 1) was determined by spectroscopic methods applied to the intact molecule and to the fragments of hydrolysis with mineral acid[5,6]. The fragments isolated under these conditions were the heterocyclic chromophore **2**, (S) proline **3**, (S) serine **4**, glycine **5** and two thiazolyl amino-acids **6** and **7** (Fig. 2).

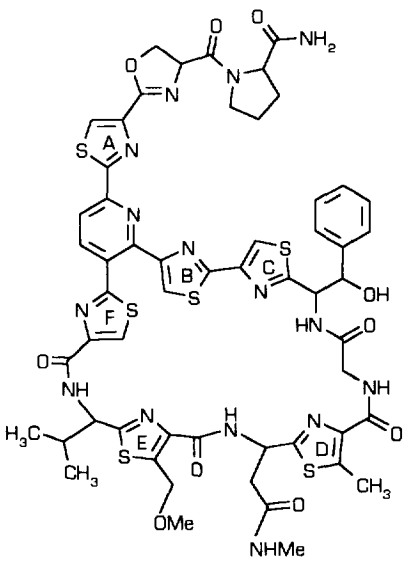


Figure 1

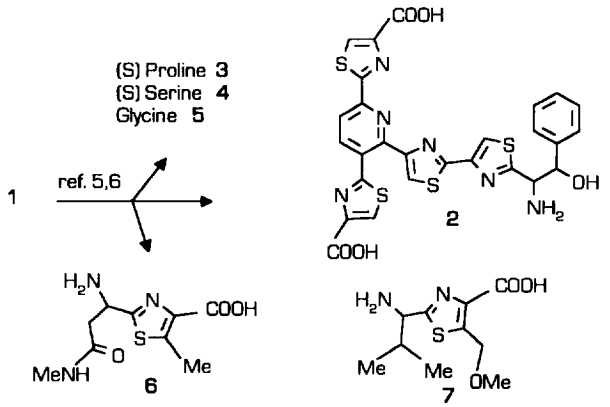


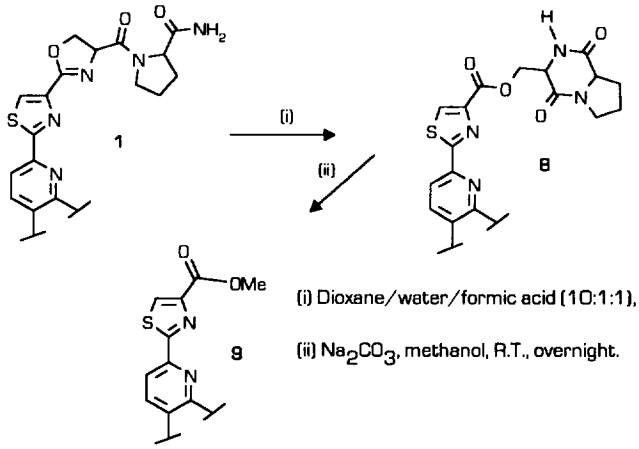
Figure 2

These thiazolyl amino-acids possess one asymmetric center, most likely derived from valine and asparagine in analogy with other thiopeptide antibiotics [7,8]. During these studies the insight into the macrocycle of MDL 62,879 **1** was limited owing to the lack of significant (MS) fragments and restricted information from the NMR due to the absence of sequential NOEs.

Here we report degradation studies on **1** which allowed the determination of the absolute configuration of the two thiazolyl amino acids and led to the revision of their sequence in the structure of the antibiotic.

RESULTS AND DISCUSSION

Chiral HPLC[9] analyses of thiazolyl amino acids **6** and **7** obtained from **1** under strong hydrolytic conditions[10] confirmed that complete racemization of amino acids occurs under those conditions. These results are in agreement with what described for other natural thiazolyl peptides[10]. Milder hydrolytic conditions produced only partial modification of the antibiotic whereas the macrocycle containing the thiazole amino acids remains untouched. In fact, acid hydrolysis of **1** in a mixture of dioxane, water and formic acid (10:1:1) yielded **8**[11] (Scheme 1) as a



Scheme 1

consequence of the opening of the oxazoline ring, N-O acyl shift and diketo piperazine formation. Methanolysis of compound **8** in the presence of Na_2CO_3 removed the diketopiperazine moiety to give the corresponding methyl ester **9**. The NMR data of the natural antibiotic **1** and the methyl ester **9** are reported in Table I. The NMR spectra of compound **9** showed no evidence of racemization as confirmed also by chiral HPLC analysis.

Table I.

	MDL 62,879[a)]	**MDL 62,879[a)]**	*Compound* **9**[b)]	*Compound* **9**[b)]
	1H-NMR	13C-NMR	1H-NMR	13C-NMR
Gly-NH	8.42(dd J=8.8,3.7 Hz)		8.46(dd J=8.8,3.7 Hz)	
Gly-α	4.31/3.82(dd 17,8.8 Hz; dd 17, 3.7 Hz)	41.0	4.28/3.79(dd 17,8.8 Hz; dd 17, 3.7 Hz)	41.0
Gly-C'		169.3		169.4
Val-NH	8.68(d 8.2 Hz)		8.69(d 8.1 Hz)	
Val-α	5.21(m)	55.2	5.21(dd 8.1,4.8 Hz)	55.2
Val-β	2.19(m)	33.8	2.17(m)	33.9
Val-γ	0.90(d 7.0 Hz)	17.7	0.88(d 7.0 Hz)	17.8
Val-γ'	0.86(d 7.0 Hz)	18.2	0.85(d 7.0 Hz)	18.3
ThiazoleD-2		165.4		165.4
ThiazoleD-4		143.5		143.6
ThiazoleD-5		140.8		140.7
ThiazoleD-OCH2	5.00(s)	67.1	4.98(s)	67.2
ThiazoleD-CH3	3.40(s)	58.4	3.39(s)	58.5
ThiazoleD-C'		161.1		161.2
Asn-NH	8.72(d 8.2 Hz)		8.69(d 8.8 Hz)	
Asn-α	5.32(m)	47.9	5.30(m)	47.9
Asn-β	2.73/1.39	37.4	2.72/1.29(dd 16.2,3 Hz) (b.s.)	37.4
Asn-γ-C'		169.6		169.6
Asn-Δ-NH	7.37(q)		7.39(q)	
Asn-ε-CH3	2.49(d)*	25.6	2.48(d 4.4 Hz)	25.7
ThiazoleE-2		168.2		168.3
ThiazoleE-4		141.9		141.9
ThiazoleE-5		139.3		139.3
ThiazoleE-CH3	2.61(s)	11.7	2.59(s)	11.8

Table I. continued

	MDL 62,879[a]	**MDL 62,879**[a]	*Compound 9*[b]	*Compound 9*[b]
	1H-NMR	13C-NMR	1H-NMR	13C-NMR
ThiazoleE-C'		161.0		161.0
ThiazoleF-2		164.5		164.6
ThiazoleF-4		149.2		149.3
ThiazoleF-5	8.58(s)	126.6	8.61(s)	126.5
ThiazoleF-C'		160.0		160.1
Pyridine-2		150.1*		150.1*
Pyridine-3		127.4		127.6
Pyridine-4	8.39(d 8.1 Hz)	141.1	8.42(d 8.1 Hz)	141.3
Pyridine-5	8.28(d 8.1 Hz)	118.4	8.30(d 8.1 Hz)	118.5
Pyridine-6		149.9**		150.0**
ThiazoleB-2		160.2		160.3
ThiazoleB-4		153.2		153.2
ThiazoleB-5	8.28(s)	122.9	8.29(s)	123.1
ThiazoleC-2		170.2		171.0
ThiazoleC-4		146.7		146.7
ThiazoleC-5	7.37(s)	116.1	7.36(s)	116.3
PheSer-NH	8.96(d 7.4 Hz)		9.04(d 7.7 Hz)	
PheSer-α	5.27(t 7.4 Hz)	58.0	5.24(t 7.3 Hz)	58.0
PheSer-β	5.04	73.6	5.00	73.7
PheSer-β-OH	5.99(d 3.7 Hz)		6.04	
PheSer-1		141.5		141.7
PheSer-2.6	7.32(m)	126.5	7.32(m)	126.6
PheSer-3,5	7.29(m)	127.7	7.29(m)	127.8
PheSer-4	7.24(m)	127.4	7.24(m)	127.4
ThiazoleA-2		168.0		167.9
ThazoleA-4		144.5		147.3
ThiazoleA-5	8.58(s)	126.6	8.78(s)	132.3
Oxazoline-C=N		159.1		
Oxazoline-α	5.24(m)	67.7		
Oxazoline-β	4.83/4.57(t 8/t 8.9 Hz)	69.15		

Table I. continued

	MDL 62,879[a)]	**MDL 62,879**[a)]	*Compound* **9**[b)]	*Compound* **9**[b)]
	1H-NMR	13C-NMR	1H-NMR	13C-NMR
Oxazoline-C'		167.6		
Proline-α	4.28(m)	59.9		
Proline-β	2.15/1.90(m, m)	29.5		
Proline-γ	1.97(m)	24.0		
Proline-Δ	4.01/3.83(m, m)	46.8		
Proline-C'		173.4		
Proline-NH2	7.32/6.91(s, s)			

a) chemical shifts in DMSO-d_6 at 308 K

b) chemical shifts in DMSO-d_6 at 300 K

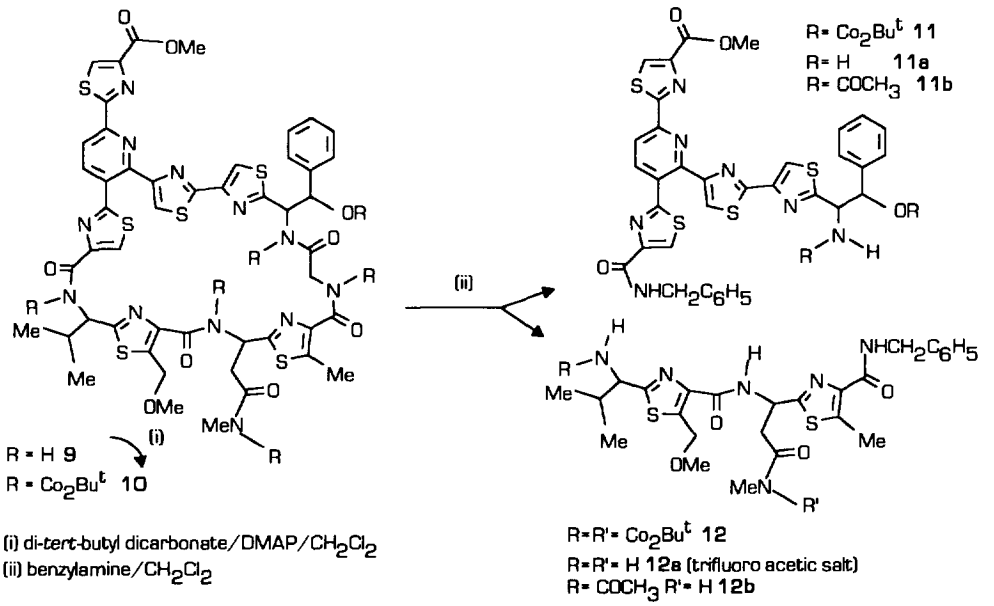
*) under DMSO signal; **) carbons 2 and 6 of the pyridine ring could not be distinguished

The degradation of compound **9** was studied with the aim of isolating the thiazolyl amino-acids in their original enantiomeric form. The methodology of Ireland[12], based on the use of singlet oxygen, failed in determining the chirality of the thiazol amino acids. When applied to **9** the starting material was recovered unmodified.

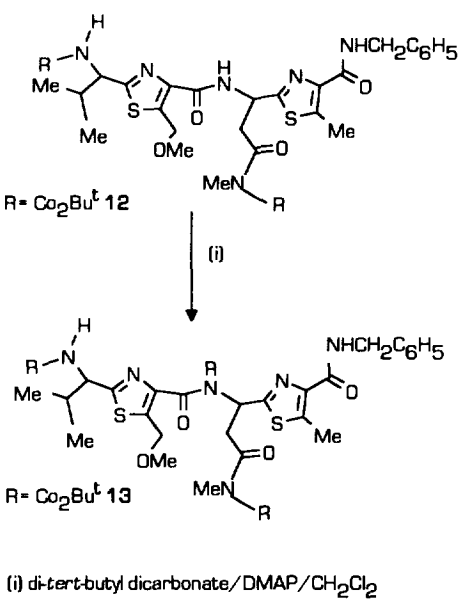
The selective opening of the macrocycle of **9** was done according to the method of Grieco *et al.*[13]. They reported that the acylation of an amide bond with di-*tert*-butyl dicarbonate results in the formation of an "activated imide" which reacts regiospecifically with nucleophiles. The selectivity of the reaction is explained by the sterical hindrance of the *tert*-butyl group leading to the breakage of the original amidic bond. Indeed, compound **9** was treated in dichloromethane solution with an excess of di-*tert*-butyl dicarbonate and in the presence of stoichiometric amounts of 4-dimethylamino pyridine to give compound **10** (Scheme 2). Six acyl residues are present in **10** as demonstrated by FAB-MS studies. Reaction of compound **10** with benzylamine at room temperature gave compounds **11** and **12** as the main reaction products. Minor reaction products were not investigated.

Compound **11** was the chromophoric fragment which is responsible for the UV absorption[6] of GE2270A. Both NMR spectra and chiral HPLC of compound **11** and of its derivatives **11a** and **11b**, suggested that compound **11** was a single enantiomer.

Compound **12** was the dipeptide of the two thiazolyl amino acids. It was a single peak in chiral HPLC. The reversed-phase HPLC of the Marfey's[14] derivative of **12a** confirmed that no racemisation occurred during the two step degradation from compound **9**. Finally, compound **12** was deprotected and acetylated to give N-acetyl **12b**. Compound **12** was submitted to a second degradation cycle, according to Grieco methodology, to obtain the individual thiazolyl amino acids. The acylated compound **13** was obtained but it continued to be resistant to the action of different nucleophiles (Scheme 3). However, compound **12** was submitted to ozonolysis of the thiazole rings followed by acid hyrolysis as described by Kobayashi *et al.*[15]. This method provided the valine and glutamic acid with the original chirality. Their 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate derivatives[16] were single peaks in HPLC analysis and coeluted with standards S amino acids derivatives. This is likely the configuration of the stereocenter in the original antibiotic **1** as the configuration was retained during the sequence of reactions.



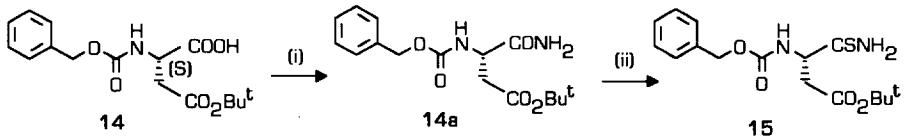
Scheme 2



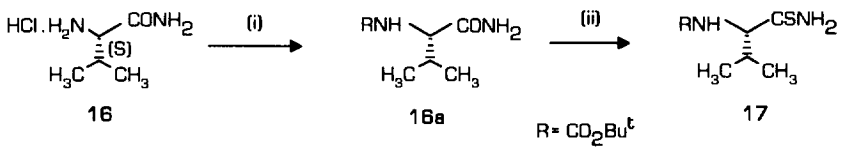
Scheme 3

To confirm the structure, the thiazole dipeptide **12** was then synthesized. The stereospecific synthesis was carried out according to Bredenkamp *et al.*[17,18] by coupling N-protected α-amino thiocarboxyamides with the corresponding 3-bromo-2-oxo esters.

The thiocarboxyamides **15** and **17** were obtained as single stereoisomers (chiral HPLC analysis) in a two steps synthesis from Z-L-aspartic acid 4-tert-butyl ester **14** (Fluka) and L-valinamide hydrochloride **16** (Novabiochem) respectively (Scheme 4).



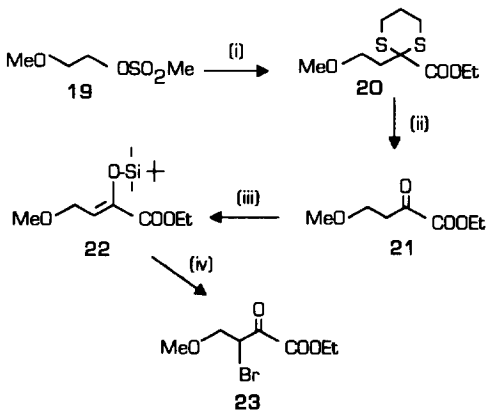
(i) isobutyl-chloro formate/N-methyl morpholine, NH_3(g).
(ii) Lawesson's reagent



(i) di-*tert*-butyl dicarbonate/triethylamine/dioxane
(ii) Lawessons's reagent

Scheme 4

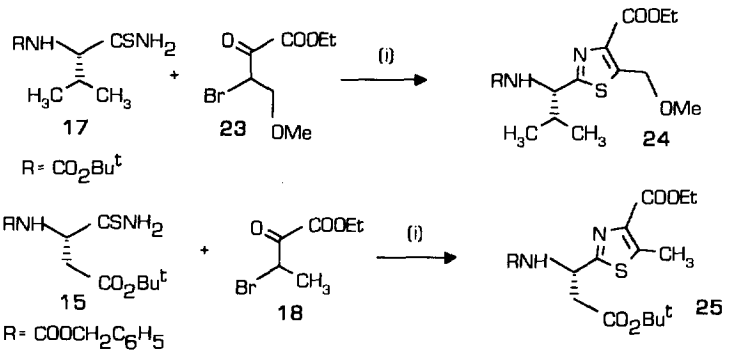
Racemic ethyl *dl*-4-methoxy-3-bromo-2-oxobutyrate **23** was obtained by alkylation of **19** with the ethyl 1,3-dithiane-2-carboxylate (Fluka), deprotection of compound **20** by catalytic amounts of cerium ammonium nitrate to give **21** and bromination of the corresponding enolsilyl ether **22** (Scheme 5) and the ethyl *dl*-3-bromo-2-oxobutyrate **18** was prepared according to the reported procedure[19].



(i) Ethyl 1,3-dithiane-2-carboxylate, sodium hydride (50%)
(ii) Sodium bromate, ceric ammonium nitrate (cat.), acetonitrile/water 7/3
(iii) *tert*-butyl-dimethylchlorosilane,DMAP, CH_2Cl_2/Pyridine 3/1
(iv) Bromine 1M/CH_2Cl_2

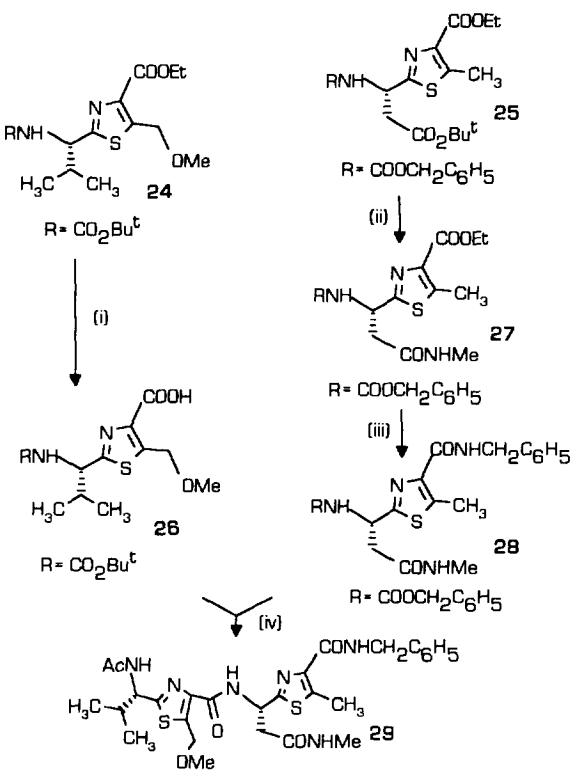
Scheme 5

The thiazole amino acids **24** and **25** were obtained (Scheme 6) as single stereoisomers (chiral HPLC analysis) and, after appropriate modifications, were coupled leading to the dipeptide **29** (Scheme 7).



(i) $KHCO_3$, dimethoxyethane then trifluoroacetic anhydride/pyridine

Scheme 6



(i) NaOH 1N/dioxane; (ii) TFA then DPPA/methylamine;
(iii) NaOH 1N/dioxane then DPPA/benzylamine;
(iv) see Experimental

Scheme 7

A comparison of the NMR spectra (Table II and Fig. 3) and the chiral HPLC profiles of compounds **29** and **12b**, revealed that the two compounds differ significantly.

Table II.

	Compound **12b**	*Compound* **12b**	*Compound* **29**	*Compound* **29**
	1H-NMR*)	13C-NMR*)	1H-NMR*)	13C-NMR*)
Acetyl-CH3	1.98(s)	23.2	2.04(s)	23.4
Acetyl-C'		169.9		169.9
Val-NH	7.86(d 9.2 Hz)		6.39(d 8.8 Hz)	
Val-α	5.15(dd 9.2,5.5 Hz)	55.9	5.09(dd 8.8,5.8 Hz)	55.9
Val-β	2.31(m)	33.4	2.23(m)	33.4
Val-γ	0.92(d 7.0 Hz)	19.4	0.88(d 6.6 Hz)	19.2
Val-γ'	0.89(d 7.0 Hz)	17.7	0.88(d 6.6 Hz)	17.9
Asn-NH	7.95(d 8.1 Hz)		9.22(d 8.4 Hz)	
Asn-α	5.42(m)	48.1	5.52(m)	48.0
Asn-β	2.84/2.67(dd 15,4/ 15,4.8 Hz)	38.2	2.97/2.80(dd 15,4.9/ 15,4.4 Hz)	38.8
Asn-γ-C'		171.2		170.6
Asn-Δ-NH	5.77(q)		5.99(q)	
Asn-delta-CH3	2.60(d 4.8 Hz)	26.2	2.61(d 4.8 Hz)	26.3
Benzyl-NH	7.90(t 6.3 Hz)		7.57(t 5.3 Hz)	
Benzyl-CH2	4.58/4.52(dd 14.7, 6.3 Hz)	43.1	4.54/4.52(dd 15, 5.3 Hz)	43.0
Benzyl-1		138.5		138.4
Benzyl-2,6	7.29(m)	127.8	7.30(m)	127.9
Benzyl-3,5	7.27(m)	128.7	7.30(m)	128.8
Benzyl-4	7.21(m)	127.4	7.24(m)	127.5
Val-Thiazole-2		169.1		168.0
Val-Thiazole-4		141.8		140.7
Val-Thiazole-5		143.5		144.7
Val-Thiazole-C'		162.0		161.6
Val-Thiazole-CH2	5.03/4.99(d 14.7 Hz)	68.3	5.0/4.98(d 15 Hz)	68.1
Val-Thiazole-CH3	3.39(s)	59.1	3.42(s)	59.2
Asn-Thiazole-2		166.6		166.2

Table II. continued

	Compound 12b	*Compound* 12b	*Compound* 29	*Compound* 29
	1H-NMR*)	13C-NMR*)	1H-NMR*)	13C-NMR*)
Asn-Thiazole-4		141.8		141.8
Asn-Thiazole-5		142.1		142.0
Asn-Thiazole-C'		162.1		162.3
Asn-Thiazole-CH3	2.66(s)	12.6	2.72(s)	112.8

*) chemical shifts in $CDCl_3$ at 280 K

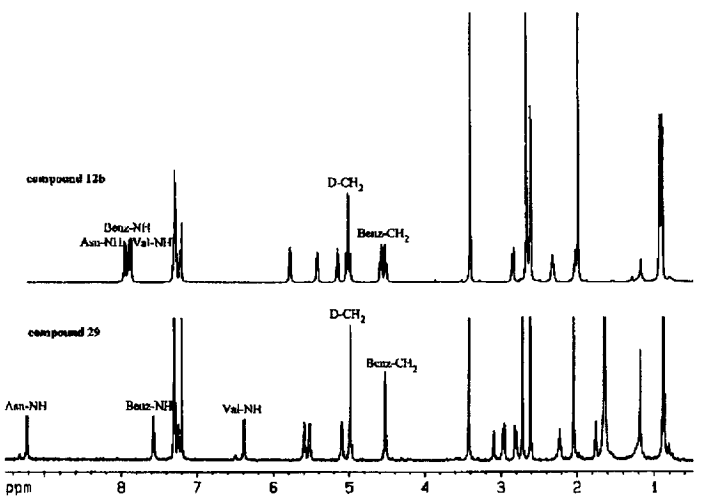


Figure 3

The amide protons of the two molecules have very different chemical shifts. A relevant difference is also observed in the coupling patterns of the signals for the methylenic protons of benzylamide and the valine thiazole which are characterized by a stronger roof effect and a higher degree of degeneration in **29** with respect to **12b**.

Therefore the sequential connectivities of the two thiazole amino acids in **29** and **12b** were investigated by NMR using the HMBC technique[20]. In the HMBC spectrum of **12b** the carbon of the acetyl group is correlated to the NH and C-α proton of the asparagine thiazole residue whereas in the synthetic compound **29** it is correlated with the NH and C-α proton of the valine thiazole residue (Fig. 4). These data are consistent with a sequence in the natural fragment **12** which is reversed with respect to the structure of **1** previously proposed.

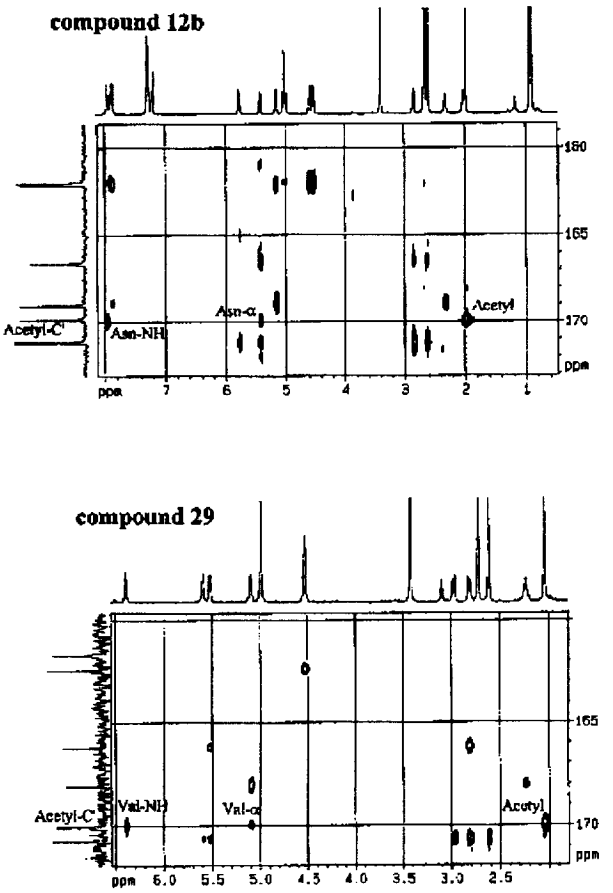
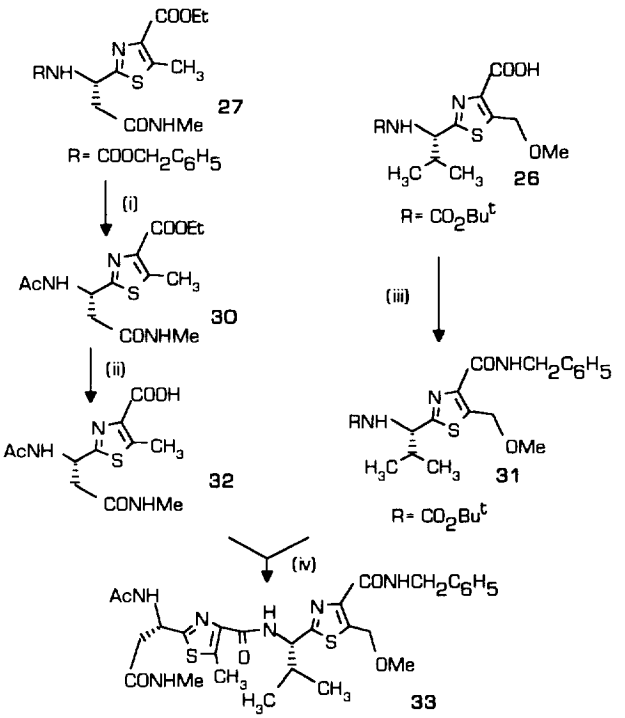


Figure 4

To confirm this finding, the thiazole dipeptide **33** with the reversed sequence was also synthesized (Scheme 8).

Compounds **33** and **12b** coeluted in chiral HPLC analysis and showed identical ^{1}H-NMR spectra. In the HMBC spectrum of all thiazole dipeptide fragments the carbonyl carbon of the acetyl group can be assigned unambigously due to the strong coupling to the methyl group (antenna function) proving that the sequence of the two thiazol amino acids is reversed with respect to the previously proposed structure.

It was not possible to obtain a correlation in the HMBC spectrum of the natural compound between the carbonyl carbon of the thiazole ring F and the proton H5 on the same ring. Such a correlation would have proved the direct connection between thiazole ring F and the amino acid asparagine or valine respectively. However, the corresponding coupling constant (although it is a coupling via three bonds) is very small (about 1 Hz) and therefore not detectable in the HMBC spectrum.



(i) TFA/thioanisole then Py/Ac_2O; (ii) NaOH 1N/dioxane ;
(iii) Py-Bop/benzylamine; (iv) see Experimental

Scheme 8

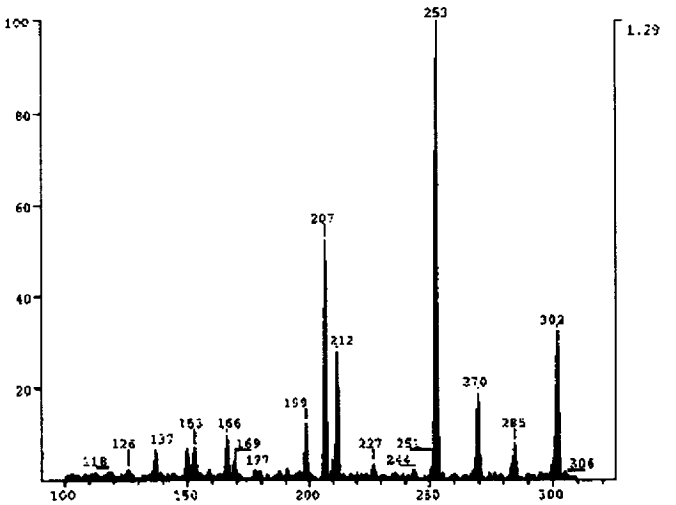
Additional evidence of the revised sequence was obtained[22] from fragments of the hydrolysis of **1** analyzed by mass spectroscopy. After two hours incubation, the HPLC profile showed several peaks whose UV spectra indicated the presence of the thiazole ring (Diode Array analysis).

The valine-thiazol-glycine **34** and the asparagine-thiazol-valine-thiazol-glycine **35** fragments were isolated from the hydrolysis mixture and their mass spectra are reported in Figures 5 and 6, respectively.

The FAB-MS spectrum of **34** showed the peak at m/z 302 corresponding to the quasi-molecular ion. FAB-MS/MS experiments were performed on the quasi molecular ion at m/z 302 and the fragmentation pattern is consistent with the losses of glycine, ammonia and methanol leading to the ions at m/z 227,285, 270 respectively. A further confirmation of the structure was given by MS/MS experiments carried out on the daughter ions produced during the fragmentation process of the parent ion at m/z 302 (Fig. 5).

4MS/MS experiments on the daughter ion at m/z 465 confirmed the structure (Fig.6). The ^{1}H-NMR spectra of the compounds **34** and **35** were in accordance with the structure suggested by mass results.

The sequence of the thiazole amino acids is thus consistent with the revised structure of the natural antibiotic as shown in Fig. 7.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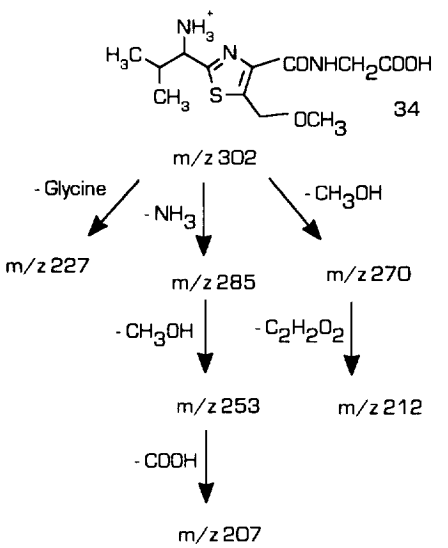
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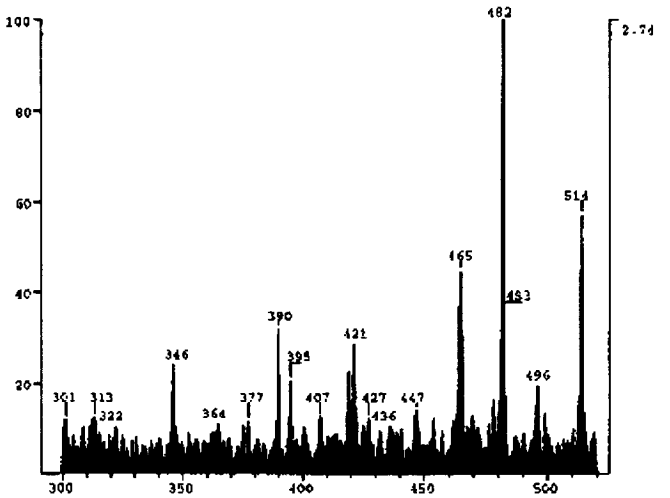
CH3
N
S
CONHCH2COOH
OCH3
34
m/z 302
- Glycine
- NH3
- CH3OH
m/z 227
m/z 285
m/z 270
- CH3OH
- C2H2O2
m/z 253
m/z 212
- COOH
m/z 207

Figure 5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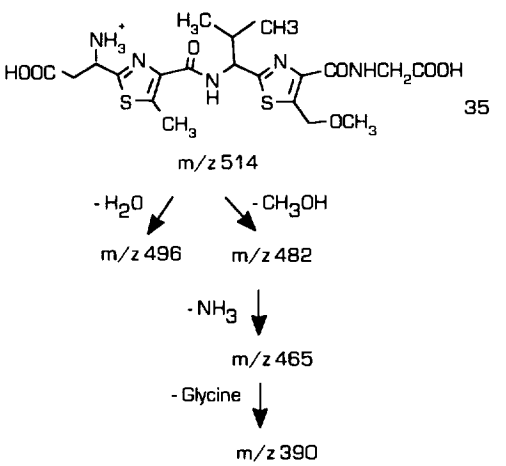
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NH3+
HOOC
O
N
H
CONHCH2COOH
CH3
OCH3
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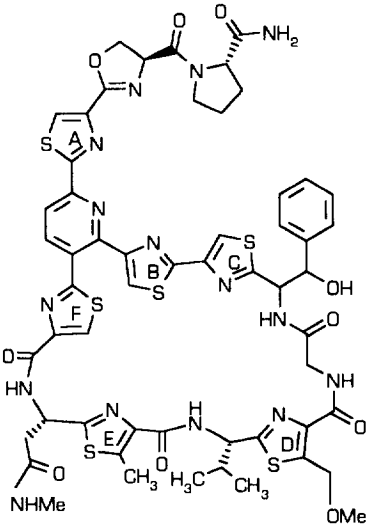
-CH3OH
m/z 496
m/z 482
-NH3
m/z 465
-Glycine
m/z 390

Figure 6



MDL 62,879 (revised structure)

Figure 7

CONCLUSION

Due to the structure of MDL 62,879, it was very difficult to assign the correct sequence by NMR or mass spectroscopy on the intact molecule. The ROESY spectrum shows only a few ROEs at 600 and 500 MHz due to the small number of protons in the molecule and provides insufficient structural information for the sequencial assignment. The two amino acids asparagine and valine are separated by a thiazole ring and therefore none of the sequential NOEs which are typical for peptides and proteins can be observed. Also between the amide proton of asparagine and H5 on the thiazole ring F no ROE is detectable which can be explained by a corresponding conformation. The results here described demonstrate the importance of the co-operation of several disciplines (synthesis, spectroscopic and chromatographic analysis) for the structure determination of complex natural antibiotics.

EXPERIMENTAL

The solvents and reagents were purified and dried by standard techuiniques. Melting points were determined with Buchi Model 510 capillary apparatus and are uncorrected.

The IR absorption spectra were recorded with I.F.S. 48 Bruker F.T.I.R. spectrophotometer.

The UV absorption spectra were recorded with a Perkin-Elmer spectrophotometer Mod. Lambda 16 (200-800 nm).

Optical rotations were measured with a Perkin-Elmer Model 241 polarimeter.

All NMR experiments were performed on a Bruker AM 500 or Bruker AMX 600 spectrometer equipped with a X32 computer. The data were processed on an external Aspect station using UXNMR software from Bruker. All of the homonuclear experiments (COSY;ROESY) were acquired with spectral widths of 11 ppm. The spectra were recorded with 512 increments in t_1 and 4096 complex points in t_2. For the COSY sectra, 32 transients were

registered for each t_1 value, while 48 transients were recorded for the ROESY spectra. The HMBC spectra were obtained using the original pulse sequence from Bax and Summers[21] . 512 FIDs with 160 scans and 2048 complex data points were acquired. Spectral widths of 11 ppm in the proton dimension and 200 ppm in the carbon dimension were used. After Fourier transformation the strong t_1 noise was reduced by a mean row subtraction using the spectrum analysis program AURELIA (Bruker).

The MS spectra were obtained with a triple stage quadrupole spectrometer TSQ 700 Finningan under the following conditions:

FAB: saddle field atom gun with Xe gas at 8kV voltage and 1 mA current, matrix NBA or thioglycerol; CI: positive ion mode, source temp. 120°C, CH_4 gas, 120 eV; EI: ion source 150°C, 70 eV. The elemental analysis was carried out with Carlo Erba Mod. 1106 equipped with Eager 200 computer. The "flash chromatography" purifications were performed as reported[23]. The reactions were monitored by TLC on Silica Gel 60 F254 (Merck) and detected by UV (254 nm).

HPLC analysis: Varian 5000 Liquid Chromatograph equipped with a 3390 A Hewlett Packard integrator. Detection UV at 254 nm. Column Lichrocart 125-4 (Merck) - Lichrospher 100 RP18 (5 μm). Mobile phase A : ammonium formate 0.05 M; mobile phase B : acetonitrile. Flow-rate : 0.7 mL/min.
Chiral HPLC: Liquid Chromatograph Hewlett Packard Mod.1090 equipped with a diode- array detector. Bakerbond® prepacked column (J.T.Baker 250 mm x 4.6 mm; 5 μm). Chiral stationary phase : DNBPG covalently linked. Mobile phase A : n-hexane ; mobile phase B : isopropyl alcohol and acetonitrile when needed.

Compound **8**. - A solution of 5 g of **1** (3.87 mmoles) in 60 ml of dioxane, 6 ml of water and 6 ml of formic acid 99% was left overnight at 80° C under stirring. After cooling, the solvent was evaporated and concentrated *in vacuo* through azeotropical removal of water with toluene. The yellow solid residue was chromatographed on silica gel ($CHCl_3$-MeOH 95:5) to give 4.07 g of pure **8** as a pale yellow solid (3.15 mmoles, yield 81%), UV (MeOH) λ_{max}= 306 nm; υ_{max}(nujol)/cm^{-1} 1725, 1655, 1410; δ_H (DMSO-d_6) 9.00 (1H, d 8 Hz), 8.69 (1H, d 8.6 Hz), 8.68 (1H, d 8 Hz), 8.61 (1H, s), 8.46 (1H, s), 8.45 (1H, d 8 Hz), 8.45 (1H, t), 8.30 (1H, s), 8.27 (1H, d 8 Hz), 7.37 (1H, q), 7.36 (1H, s), 7.33-7.26 (4H, m), 6.01 (1H, d 4.3 Hz), 5.28 (1H, m), 5.24 (1H, t 7.3 Hz), 5.21 (1H, dd 8.3, 4.6 Hz), 5.01 (1H, t 5.6 Hz), 4.98 (2H, s), 4.68 (1H, dd 9.6, 2.3 Hz), 4.53 (2H, m), 4.35-4.28 (2H, m), 3.77 (1H, dd 16.9, 4.0 Hz), 3.55 (1H, m), 3.39 (3H, s), 2.70 (1H, dd 16.6, 3.0 Hz), 2.59 (3H, s), 2.47 (3H, d 4.6 Hz), 2.23-2.13 (2H, m), 1.91-1.80 (3H, m), 1.35 (1H, d 12.9 Hz), 0.88 (3H, d 7.0 Hz), 0.84 (3H, d 7.0 Hz); $[\alpha]_D^{25}$ = +126.1 (c = 1 $CHCl_3$-MeOH 9:1); FAB-MS 1291 (MH^+, 100%), *Found*: C, 52.05%, H, 4.34, N.*15.11; calculated for* $C_{56}H_{54}N_{14}O_{11}S_6$: C, 52.09%, H, 4.18, N, 15.9.; t_R (HPLC): 7.88 min., mobile phase B 44%.

Compound **9**.- A suspension of 10 g of **8** (7.75 mmoles) and 1.18 g of sodium carbonate in 200 ml of dry methanol was stirred at R.T. overnight. The mixture was filtered and the filtrate was concentrated *in vacuo*. The crude material was purified on silica gel by "flash chromatography" ($CHCl_3$ - MeOH 98:2) producing 6.36 g of pure **9** as a yellow solid (5.58 mmoles, yeld 72%), m.p. 190°C (dec.). υ_{max}(nujol)/cm^{-1} 3352, 1724, 1661, 1242-1217, 1096; FAB-MS m/z 1139(MH^+, 100%),; 1032 (35).; ^1H and ^{13}C NMR see Table 1; $[\alpha]_D^{25}$ = +163,3° (c 1, $CHCl_3$); *Found*: C, 50.54%, H, 4.06, N. *14.21; calculated for* $C_{49}H_{46}N_{12}O_9S_6$: C, 51.65%, H, 4.06, N, 14.75.; t_R (HPLC) : 23.1 min., gradient profile time(min.) 0 (B 38%), 5 (B 38%), 6 (B 45%), 10 (B 45%), 11 (55%), 20 (55%), 21 (75%).

Compound **10.** - To a stirred solution of **9** (5 g , 4.39 mmoles) in 80 ml of methylene chloride (ethanol free -Merck) were added at room temperature and under argon 0.63 ml of triethylamine (4.4 mmoles) and 538 mg of 4-

dimethylaminopyridine. 24.1 g (110.44 mmoles) of di-tertbutyl dicarbonate were then added in portions within 10 min. After 27 hours the reaction mixture was concentrated *in vacuo*, redissolved in 80 ml of methylene chloride (ethanol free) and to the solution triethylamine (0,63 ml) and di-tertbutyl dicarbonate (12g, 54.98 mmoles) were added. After stirring over night the solvent was removed, the residue taken into 100 ml of chloroform, washed with water (100 ml), dried over sodium sulfate and filtered through silica gel ($CHCl_3$ - MeOH 99:1) yielding 7.0 g of **10** (4.02 mmoles, yield 91.7%), which was used without further purification for the following reaction. FAB-MS m/z 1739 (MH^+, 60%), 1639 (100), 1539 (75), 1439 (28), 1139 (25). t_R (HPLC) 56.6 min., gradient profile time(min.) 0 (B 70%), 18 (70%), 19 (85%).

Compounds **11** *and* **12**. - A solution of 7 g of compound **10** (4.02 mmoles) and 3.2 ml of benzylamine (29.01 mmoles) in 80 ml of methylene chloride was stirred for 66 h at R. T. then washed with water (2 x 40 ml), dried on sodium sulfate and concentrated *in vacuo*. The crude material was purified by flash column chromatography on silica gel (hexane-ethyl acetate 7:3) producing 3.09 g of **11** (3.3 mmoles) and 1.31 g of **12** (1.72 mmoles).

Compound **11**: t_R (HPLC) : 14.94 min. gradient profile B; FAB-MS m/z 938 (MH^+, 70%),764 (58), 720 (98), 631 (100), δ_H (DMSO-d_6) 9.05 (1H, t 6.27 Hz), 8.75 (1H, d 4.2 Hz), 8.37 (1H, s), 8.35 (1H, d 8.2 Hz), 8.31 (1H, s), 7.9 (1H, s), 7.87 (1H, d 8.9 Hz), 7.45-7.22 (11H, m), 6.0 (1H, d 9.3 Hz), 5.25 (1H, t 9.0 Hz), 4.45 (2H, d 6.75 Hz), 3.9 (3H, s), 1.44-1.20 (18H, m).
Chiral HPLC : t_R = 19.7 min. mobile phase A: n-Hexane, mobile phase B: isopropyl alcohol, gradient profile: time(min) 0 (B 40%); time(min.) 25 (B 55%), flow 1 ml/min.

Compound **12** : FAB-MS m/z 759 (MH^+, 100%), 715 (10), 659 (25); t_R (HPLC) : 12.03 min., gradient profile as for **11**; δ_H ($CDCl_3$) 7.85 (1H, d 8.9 Hz), 7.75 (1H, t 6.1 Hz), 7.35-7.26 (5H, m), 5.9 (1H, m), 5.3 (1H,m), 5.18 (1H, dd 5.1, 8.9 Hz), 5.10 (2H, dd 14.6, 26.2 Hz), 4.6 (2H, m), 3.8 (1H, d 15.4 Hz), 3.45 (3H, s), 3.43 (1H, dd 5.2, 17.6 Hz), 3.1 (3H, s), 2.75 (3H, s), 2.4 (1H, m), 1.5-1.4 (18H, m), 1.0 (3H, d 6.8 Hz), 0.97 (3H, d 6.8 Hz); $[\alpha]_D^{25}$ = +43.7° (c 0.6, $CHCl_3$); *Found*: C, 56.92%, H, 6.67, N, 10.83, *calculated for* $C_{36}H_{50}N_6O_8S_2$: C, 56.97%, H, 6.61, N, 11.08.
Chiral HPLC : t_R = 33.7 (min.), gradient profile time(min.) 0 (B 0%), 25 (B 10%), 30 (15%), 40 (20%), flow : 1 ml/min.

Compound **11a**. - 2.6 g of **11** (2.77 mmoles) was left for 5 min. in 3 ml of trifluroacetic acid at R.T. Elimination of the trifluoroacetic acid *in vacuo* yielded a solid that was dissolved in 20 ml of chloroform, washed with a saturated solution of sodium bicarbonate (20 ml). The residue obtained after drying with sodium sulfate and concentration *in vacuo* was purified by flash chromatography on silica gel ($CHCl_3$- MeOH 97:3) to give 1.2 g of **11a** (1.62 mmoles, yield 58.7%) An analytical sample was obtained by crystallization from chloroform - isopropyl alcohol: m.p. : 174.5 (dec:); UV (MeOH) λ_{max}=304 nm; υ_{max}(nujol)/cm^{-1} 3360, 2914, 2856, 1722, 1657, 1456; δ_H (DMSO-d_6) 9.06 (1H, t 6.2 Hz), 8.74 (1H, s), 8.73 (1H,d 8.4 Hz), 8.37 (1H, s), 8.34 (1H, d 8.2 Hz), 8.26 (1H, s), 7.74 (1H, s), 7.28-7.19 (10H, m), 5.78 (1H,d 4.2 Hz), 4.95 (1H, t), 4.46-4.28 (4H, m), 3.89 (3H, m); FAB-MS m/z 738 (MH^+, 100%), 720 (15), 631 (25), 518 (39); $[\alpha]_D^{25}$ = -12.4° (c 1, $CHCl_3$); *Found*: C, 56.67%, H, 3.71, N, 13.10, S, 17.12; *calculated for* $C_{35}H_{27}N_7O_4S_4$: C, 56.96%, H, 3.69, N, 13.29, S, 17.38; t_R (HPLC) : 13.2 min., gradient profile: time(min) 0 (B 44%), 5 (B 44%), 6 (65%), 25 (85%).

Compound **11b**. - 300 mg of **11a** (0.408 mmoles) in 3 ml of pyridine were stirred overnight in the presence of 0.2 ml of acetic anydride and catalytic amounts of 4-dimethylaminopyridine. The reaction mixture was diluted with ice-cold hydrochloric acid 1N (20 ml) and extracted with methylene chloride (3x10 ml). The organic phases were washed with a solution of sodium bicarbonate (20 ml), water (20 ml) and dried over sodium sulfate. Evaporation of the solvent gave a solid which was washed with ethyl ether obtaining 288 mg of **11b** (0.350 mmoles, yield 85%); An analytical sample was obtained by crystallization from ethyl acetate; m.p. 240°C; UV (MeOH) λ_{max}=305 nm; $\upsilon_{max}(CDCl_3)/cm^{-1}$ 3420, 1743, 1670; t_R (HPLC) : 18.4 mobil phase B 50%; FAB-MS m/z 822 (MH^+, 100%), 762 (25%), 720 (40%), 673 (40%), 630 (20%); δ_H (DMSO-d_6) 9.1 (1H, t 6.2 Hz), 8.80 (1H, d 8.75 Hz), 8.70 (1H, m), 8.37 (1H, s), 8.35 (1H, d 8.2 Hz), 8.30 (1H, s), 7.90 (1H, s), 7.28 (11H, m), 6.20 (1H, d 8.0 Hz), 5.60 (1H, t 8.1 Hz), 4.50 (2H, d 6.3 Hz), 3.9 (3H, s), 2.0 (3H, s), 1.8 (3H, s); $[\alpha]_D^{25}$ = -31.6° (c 0.28, $CHCl_3$); *Found*: C, 57.01%, H, 3.97, N, 11.79, S, 14.93; *calculated for* $C_{39}H_{31}N_7O_6S_4$: C, 57.00%, H, 3.80, N, 11.93, S, 15.5.

Compound **12a.** - 30 mg of compound **12** (0.039 mmoles) were treated at room temperature with 0.3 mL of trifluoroacetic acid. After 5 min. the solvent was evaporated *in vacuo* and the residue was washed with ethyl ether yielding 21 mg (0.031 mmol) of the corresponding trifluoroacetate salt, FAB/MS : m/z 559 (M^+); δ_H (DMSO-d_6) 8.74 (1H, t 6.1), 8.58 (3H, m), 8.44 (1H, d 8.8 Hz), 8.17 (1H, m), 7.31 (5H, bs), 7.24 (1H, m), 5.09 (1H, q 7.0, 8.6 Hz), 5.04 (1H, m), 4.98 (2H, q), 4.43 (2H, m), 3.34 (3H, s), 2.86 (2H, m), 2.72 (3H, s), 2.60 (3H, d 4.5 Hz), 2.45 (1H, m), 0.98 (3H, d 6.7 Hz), 0.96 (3H, d 6.7 Hz); a sample of compound **12a** was derivatized with the Marfey's reagent as described[14]; t_R (HPLC) (column ultrasphere ODS Beckman 25cm x 4.6) 40.08, mobil phase A: triethylamine phosphate 50 mM (pH=3) / acetonitrile 95:5 (v/v), mobile phase B acetonitrile, gradient profile time(min.) 0 (B 0%), 80 (B 80%) flow rate 0.5 ml/min.

Compound **12b**. - 200 mg of **12** (0.263 mmoles) were treated at R.T. with 2 ml of trifluoroacetic acid for 5 min. The trifluoroacetic acid was evaporated *in vacuo*, the crude solid material washed with ethyl ether and left stirring overnight in 4 ml of pyridine, catalitic amount of 4-dimethylamino pyridine and 0.2 ml of acetic anhydride. Methylene chloride (20 ml) was added and the solution washed with water (2 x 20 ml) The residue obtained after drying with sodium sulfate and concentration *in vacuo* through azeotropical removal of pyridine with toluene was chromatographaed on silica gel ($CHCl_3$-MeOH 95:5) to give 100 mg of pure **12b** (0.16 mmoles, yield 63%) as an amorphous solid, $\upsilon_{max}(CDCl_3)/cm^{-1}$ 3404, 2966, 2932, 1666, 1549, 1504; t_R (HPLC) 12.8 min. mobile phase B 30%; FAB-MS m/z 601 (MH^+, 100%), 569 (61); ^1H and ^{13}C NMR see Table II; $[\alpha]_D^{25}$ = -32.1° (c 0.6, $CHCl_3$); *Found*: C, 55.85%, H, 5.87, N, 13.7, *calculated for* $C_{28}H_{36}N_6O_5S_2$: C, 56.0%, H, 6.0, N, 14.0; chiral HPLC: t_R 15.96 mobile phase B 30%, flow 1.5 ml/min.

Compound **13**. - A solution of 480 mg **12** (0.63 mmoles) in 10 ml methylene chloride (ethanol free) was treated with 78 mg of 4-dimethylaminopyridine (0.63 mmoles), 0.09 ml of triethylamine (0.63 moles) and 413 mg of di-tert-butyl dicarbonate (1.89 mmoles). After 24 h the solvent was evaporated, the residue dissolved in 10 ml of methylene chloride and left to react with the same amounts of triethylamine and di-tertbutyl dicarbonate as above. After 3 days the reaction underwent to the same process and then left stirring overnight.
The "work up" and the purification steps were made as reported for compound **10** to give 290 mg (0.33 mmoles, yield 53.6%) of **13**, t_R (HPLC) : 25.2 gradient profile time(min.) 0 (B 70%) 18(B 70%) 19 (B 85%); FAB/MS m/z 859 (MH^+, 100%); δ_H ($CDCl_3$) 7.9 (1H, d 8.8 Hz), 7.8 (1H, t 6.1 Hz), 7.35-7.25 (5H, m), 6.15 (1H, t 6.6 Hz), 5.20 (1H, dd 5.2, 8.8 Hz), 5.10 (2H, q 14.6, 24.0 Hz), 4.60 (1H, dq 6.3, 14.9, 21.2 Hz), 4.15 (1H, dd 6.4, 17.4 Hz), 3.60 (1H, dd 6.4, 17.4 Hz), 3.40 (3H, s), 3.15 (3H, s), 2.75 (3H, s), 2.4 (1H, m), 1.6-1.4 (27H, m), 1.0

(3H, d 6.7 Hz), 0.95 (3H, d 6.7 Hz).

Compound **14a**. -To a solution of Z-L-aspartic acid 4-tert-butyl ester **14** (FLUKA) (2.52 g; 7.4 mmoles) in 20 ml of dry dimethoxyethane at -15°C under argon were added 0.822 ml of N-methyl morpholine (7.4 mmoesl) and 1 ml of isobutylchloroformate (7.4 mmoles). After 15 min. gaseous ammonia was bubbled through the suspension for 5 min. at -15°C. and for 15 min at room temperature. The reaction was then diluted with water (50 ml) and extracted with chloroform (2x15ml). The organic phase was dried with sodium sulfate and concentrated *in vacuo*. The solid residue was washed with a mixture of hexane/ether (2:1) to give 2.16 g of compound **14a** (6.7 mmoles, yield 90%); m.p. = 87-90°C; $\upsilon_{max}(CDCl_3)/cm^{-1}$ 3526-3493, 3412, 1699; δ_H ($CDCl_3$) 7.35 (5H, m), 6.47 (1H, bs), 5.98 (1H, bs), 5.49 (1H, bs), 5.14 (2H, s), 4.5 (1H, bs), 2.92 (1H, dd 4.3, 17.1 Hz), 2.61 (1H, dd 6.6, 17.1 Hz), 1.4 (9H, s); $[\alpha]_D^{25}$ = +28.8° (c 1, $CHCl_3$); *Found*: C, 59.71%, H, 6.82, N, 8.74, *calculated for* $C_{16}H_{22}N_2O_5$: C, 59.61%, H, 6.83, N, 8.69.

Compound **15**. - A mixture of 2 g of **14a** (6.2 mmoles) and 1.25 g of Lawessons's reagent (3.1 mmoles) in 25 ml of dry dioxane was stirred under argon for 6 h at room temperature. Filtration of the precipitate and concentration of the filtrate *in vacuo* afforded a residue which was diluted with ice-cold water (50 ml) and extracted with ether (3x15 ml). The organic phase was then washed with sodium bicarbonate solution (20 ml),dried with sodium sulfate and concentrated *in vacuo*. The residue was purified by flash chromatograpy on silica gel (hexane/ethyl acetate 3:2) to give 1.65 g of pure **15** (4.8 mmoles, 77.4%); υ_{max}(neat)/cm^{-1} 3310-3206, 1771, 1622, 1506; δ_H ($CDCl_3$) 8.0 (1H, bs), 7.51 (1H, bs), 7.34 (5H, m), 6.1 (1H, m), 5.13 (2H, t 12.2 Hz), 4.81 (1H,m), 3.07(1H, dd 4.5, 16.9 Hz), 2.76 (1H, dd 7.2, 16.9 Hz), 1.43 (9H, s); $[\alpha]_D^{25}$ = +11.45° (c 0.91, $CHCl_3$); *Found*: C, 56.14%, H, 6.50, N, 7.81, *calculated for* $C_{16}H_{22}N_2O_4S$: C, 56.78%, H, 6.50, N, 8.28.

Compound **16a**.- To a solution of 2 g of L-valinamide hydrochloride **16** (NOVABIOCHEM) (13.15 mmoles) in 20 ml of dry dioxane at 0°C were added 4.8 mL of triethylamine (34.48 mmoles) and 4.2 g of di tert-butyl dicarbonate (18.9 mmoles). The reaction mixture was then stirred at R.T. for one hour, filtered and concentrated *in vacuo*. The residue was dissolved in 20 ml of chloroform, washed with water (20 ml), dried on sodium sulfate. The solid material obtained after concentration *in vacuo* was washed with ethyl ether and dried under vacuum at room temperature providing 2 g of **16a** (9.25 mmoles, yield 70.4%); m.p. 160-161°C; CI/MS m/z 217 (MH^+, 100%), 161 (30); δ_H (DMSO-d_6) 7.2 (1H, bs), 6.95 (1H, bs), 6.45 (1H, d 8.8 Hz), 3.75 (1H, m), 1.9 (1H, m), 1.4 (9H, s), 0.85 (3H, d 6.7 Hz), 0.81 (3H, d 6.7 Hz); $[\alpha]_D^{25}$ = -4.73° (c 0.66, $CHCl_3$).

Compound **17.** - A mixture of 1.75 g of **16a** (8.1 mmoles) and 1.63 g of Lawessons's reagent (4.03 mmoles) in 16 ml of dry dioxane was stirred for 20 h at R.T. under argon. More Lawessons's reagent was added (0.8 g , 1.97 mmoles) and the reaction mixture was stirred overnight. The reaction mixture was "worked up" as for compound **15**. Purification by flash chromatography (hexane/acetone 3:1) gave a solid that was crystallized from hexane providing 1.53 g of **17** (6.59 mmoles, yield 81%); m.p. 112-113°C; FAB-MS m/z 232 (MH^+, 26%), 172 (40), 116 (66), 85 (100); δ_H ($CDCl_3$) 7.95 (1H, bs), 7.60 (1H, bs), 5.25 (1H, bs), 4.2 (1H, m), 2.20 (1H, bs), 1.40 (9H, s), 0.98 (3H, d 6.9 Hz), 0.97 (3H, d 6.9 Hz); $[\alpha]_D^{25}$ = -43.48° (c 0.7, $CHCl_3$); *Found*: C, 51.47%, H, 8.38, N, 11.97, *calculated for* $C_{10}H_{20}N_2O_2S$: C, 51.69%, H, 8.67, N, 12.05; $[\alpha]_D^{25}$ = -43.48° (c 0.7, $CHCl_3$).

Compound **19**. - To a solution of 20 ml of 2-methoxy ethanol (Aldrich) (253.6 mmoles) and 53 ml of triethylamine (380.4 mmoles) in 100 ml of dichloromethane (ethanol free) at -15°C, a solution of 21.67 ml of methanesulfonyl

chloride (278.96 mmoles) in 50 ml of dichloromethane (ethanol free) was added. After two hours the reaction mixture was washed with water (200 ml), dried on sodium sulfate and concentrated *in vacuo*. The residue was distilled to give 32.62 g of **19** (211.8 mmoles, yield 83.5%); b.p. 70°C (0.3 mmHg); υ_{max}(neat)/cm^{-1} 3082, 1352, 1173; δ_H ($CDCl_3$) 4.34 (2H, t 4.5 Hz), 3.64 (2H, t 4.5 Hz), 3.38 (3H, s), 3.03 (3H, s).

Compound **20**. - 30 g of **19** (194.8 mmoles) and 18.73 g of ethyl 1,3-dithiane-2-carboxylate (97.4 mmoles) (Fluka) in 36 ml of dry dimethylformamide were added to a stirred suspension of 5.61 g of sodium hydride (50% mineral oil, 116.8 mmoles) in 160 ml of dry benzene at 5°C under argon atmosphere. After 30 min at 5°C and 4 h at R.T. the reaction was washed with phosphate buffer solution (100 ml ; pH= 5.3), 100 mL of saturated sodium chloride solution,dried on sodium sulfate and concentrated *in vacuo*. Purification by flash chromatography on silica gel (hexane/ether 3:1) gave 19.97 g of **20** (79.88 mmoles, yield 82%); υ_{max}(neat)/cm^{-1} 2978-2829, 1722, 1219, 1115; FAB-MS m/z 250 (M^+, 8%), 177 (40), 145 (12), 45 (100%); δ_H ($CDCl_3$) 4.25 (2H, q 7.0, 14.3 Hz), 3.59 (2H, t 6.9 Hz), 3.32 (3H, s), 3.27 (2H, m), 2.66 (2H, m), 2.33 (2H, t 6.9 Hz), 2.15 (1H, m), 1.88 (1H, m), 1.33 (3H, t 7.1 Hz); *Found*: C, 48.93%, H, 7.25, S, 25.07, *calculated for* $C_{10}H_{18}O_3S_2$: C, 48.04%, H, 7.24, S, 25.61.

Compound **21**. - To a stirred solution of 9 g of **20** (36 mmoles) and 16.3 g of sodium bromate (108 mmoles) in 150 ml of acetonitrile/water (7:3), 198 mg of ceric ammonium nitrate (0.36 mmoles) were added at 0°C. After stirring at R.T.for 1.5 h the starting material disappeared (TLC: hexane/ether 1:1). The reaction was diluted with a solution of sodium sulfite (50 mL) and extracted with methylene chloride (3x70 ml). The organic phase was washed with a solution of sodium sulfite (100 ml), saturated sodium chloride solution (100 ml), dried on sodium sulfate and concentrated. The residue was distilled to give 4.03 g of compound **21** (25.2 mmoles, yield 70%); b.p. 57-58°C (0.1 mmHg); υ_{max}(neat)/cm^{-1} 2986-2833, 1730, 1265, 1117; EI/MS m/z 161 (MH^+, 5%), 129 (10%), 87 (28%), 45 (100%); δ_H ($CDCl_3$) 4.31 (2H, q 7.0, 14.3 Hz), 3.70 (2H, t 6.1 Hz), 3.32 (3H, s), 3.07 (2H, t 6.1 Hz), 1.35 (3H, t 7.0 Hz).

Compound **22**. - 2.82 g of tert-butyl-dimethylchlorosilane (18.74 mmoles) were added to a stirred solution of 1.5 g of **21** (9.37 mmoles) and 1.71 g of 4-dimethylamino pyridine (14.05 mmoles) in 20 ml of methylene chloride (ethanol free)/pyridine (3:1). After 24 h the reaction was washed with 10 ml of cold hydrochloric acid 1N, saturated sodium chloride solution (10 ml), dried on sodium sulfate and concentrated *in vacuo*. The residue was rapidly chromatographated on silica gel to give 1.71 g of **22** (6.24 mmoles, yield 66.5%); R_f = 0.35 (hexane/ethyl ether 9:1); δ_H ($CDCl_3$) 6.10 (1H, t 6.4 Hz), 4.21 (2H, q 7.0, 14.2 Hz), 4.15 (2H, d 6.4 Hz), 3.35 (3H, s), 1.30 (3H, t 7.0 Hz), 0.96 (9H, s), 0.17 (6H, s).

Compound **23**. - 12.8 ml of bromine 1M in methylene chloride were added to a solution of 3.51 g of **22** (12.8 mmoles) in 36 ml of methylene chloride (ethanol free) at -50°C. After stirring over night at room temperature 1 mL of bromine 1M in methylene chloride was added at 0°C and the reaction was stirred for additional 7 h at R.T. The solvent was concentrated *in vacuo* and the residue distilled to give 1.72 g of **23** (7.23 mmoles, yield 56.4%) as a pale yellow oil; b.p. 75-77°C (0.2 mmHg); υ_{max}($CDCl_3$)/cm^{-1} 2988-2833, 1732, 1267; CI/MS m/z 238 (M^+, 100%), 208 (8%), 161 (30%), 129 (10%); δ_H ($CDCl_3$) 5.12 (1H, dd 6.0, 7.6 Hz), 4.37 (2H, q 7.0, 14.3 Hz), 3.98 (1H, dd 7.7, 10.2 Hz), 3.80 (1H, dd 6.0, 10.6 Hz), 3.41 (3H, s), 1.38 (3H, t 7.0 Hz).

Compound **24**. - A suspension of 172 mg of finely powdered potassium hydrogen carbonate (1.72 mmoles), 100 mg of **17** (0.431 mmol) and 309 mg of **23** (1.29 mmoles) in 2 ml of dry dimethoxyethane was stirred at room

temperature under argon for 24 h 0.3 ml of pyridine (3.68 mmoles) and 0.24 ml of trifluoroacetic anhydride (1.75 mmoles) in 0.5 ml of dry dimethoxyethane were added at 0°C. After stirring at R.T for 30 min., the solvent was evaporated and the residue dissolved in 10 ml of chloroform. The organic phase was washed with water (3 mL), dried on sodium sulfate and concentrated *in vacuo*. The crude was purified first on silica gel (hexane/ethyl acetate 3:1) and then on aluminium oxide (hexane/ethyl acetate 3:1) to give 85 mg of **24** (0.23 mmoles, yield 53%); t_R (HPLC) : 30.7 gradient profile time(min.) 0 (B 40%), 15 (B 40%), 18 (B 60%), 22 (B 60%), 23 (B 80%); m.p. 60-61°C; υ_{max}(nujol)/cm^{-1} 3341; 2951-2853, 1705, 1682; FAB-MS m/z 373 (MH^+, 20%), 317 (100), 273 (80), 256 (40); δ_H ($CDCl_3$) 5.27 (1H, bs), 4.93 (2H, q 14.8, 18.1 Hz), 4.83 (1H, m), 4.38 (2H, q 7.0, 14.1 Hz), 3.46 (3H, s), 2.36 (1H, m), 1.39 (9H, s), 1.37 (3H, t 7.09 Hz), 0.95 (3H, d 6.7 Hz), 0.88 (3H,d 6.7 Hz); $[\alpha]_D^{25}$ = -36.1° (c 0.22, $CHCl_3$); *Found*: C, 55.10%, H, 7.49, N, 7.43, *calculated for* $C_{17}H_{28}N_2O_5S$: C, 54.81%, H, 7.52 N, 7.52; chiral HPLC : t_R 21.71 min. mobile phase B 1%:

Compound **25**. - A suspension of 726 mg of finely powdered potassium hydrogen carbonate (7.26 mmoles), 306 mg of **15** (0.908 mmoles) and 567 mg of **18** (2.72 mmoles) in 10 ml of dry dimethoxyethane was stirred under argon for 24 h at R.T. 0.62 ml of pyridine (7.7 mmoles) and 0.51 ml of trifluoroacetic anhydride (3.68 mmoles) in 2 ml of dry dimethoxyethane were added at 0°C. After stirring at R.T. for 1.5 h the solvent was concentrated *in vacuo*, the residue dissolved in 15 ml of chloroform and the organic phase washed with water (10 mL). The crude material obtained after drying with sodium sulfate and concentration in vacuo was purified by flash chromatography on silica gel to give 330 mg of pure **25** (0.736 mmoles, yield 81%); t_R (HPLC) : 38.7 min. gradient profile time(min.) 0 (B 40%), 31 (B 40%), 32 (B 60%); υ_{max}(neat)/cm^{-1} 3337, 3090-2980, 1713; FAB-MS m/z 449 (MH^+, 30%), 393 (45), 349 (38), 285 (55), 257 (90), 239 (100), 213 (90); δ_H ($CDCl_3$) 7.29 (5H, m), 6.27 (1H, bs), 5.30 (1H, m), 5.10 (2H, q), 4.34 (2H, m), 3.05 (1H, dd 4.8, 16.3 Hz), 2.89 (1H, dd 5.2, 16.3 Hz), 2.66 (3H, s), 1.35 (12H, m); $[\alpha]_D^{25}$ = -10.6° (c 0.6, $CHCl_3$); *Found*: C, 58.73%, H, 6.16, N, 6.06, *calculated for* $C_{22}H_{28}N_2O_6S$: C, 58.91%, H, 6.29 N, 6.25; chiral HPLC : t_R 29.03 min. mobile phase B 1.%, flow 0.5 ml/min.

Compound **26**. - A solution of 94 mg of **24** (0.25 mmoles) in 4 ml of dioxane/water (8:2) and 0.5 ml of sodium hydroxide 1N was stirred at R.T. for 5h. The reaction mixture was diluted with water (2.5 ml), acidified with hydrochloric acid at pH=1 and extracted with ethyl acetate (3x3 mL). The organic phase was washed with with water (5 ml), dried on sodium sulfate and concentrated *in vacuo* to give 86 mg of 26 (0.25 mmoles, yield 100%) which was used without further purification; t_R (HPLC) : 6.11 min. gradient profile as for compound **24**; υ_{max}($CDCl_3$)/cm^{-1} 3441, 2968, 2934, 2874, 1753, 1715, 1497, 1369; δ_H ($CDCl_3$) 5.15 (1H, m), 5.01 (2H, q), 4.85 (1H, m), 3.51 (3H, s), 2.31 (1H, m), 1.46 (9H, bs), 1.00 (3H, d 6.7 Hz), 0.94 (3H, d 6.7 Hz).

Compound **27**. - 240 mg of **25** (0.53,5 mmoles) were left in 3 ml of trifluoroacetic acid at R.T. under argon for 50 min. After evaporation of the trifluoroacetic acid the residue was washed with hexane/ether (1:1) giving 200 mg (0.51 mmoles) of the corresponding acid as a white solid that was dissolved in 4 mL of dimethylformamide. To this solution 0.106 ml of triethylamine (0.765 mmoles) and 154 mg of diphenylphosphorylazide (DPPA) (0.561 mmoles) were added. After stirring for 2 h. at R.T. a solution of 51.6 mg of methylamine hydrochloride (0.765 mmoles) and 0.106 ml of triethylamine in 0.3 ml of water was added. After 50 min. the solution was diluted with water (30 ml) and extracted with ethyl acetate (2x 30 ml). The organic phase was washed with saturated solution of sodium hydrogen carbonate (30 ml), dried with sodium sulfate and concentrated *in vacuo*. The residue was purified by flash chromatography on silica gel ($CHCl_3$/Me0H 98:2) to give 175 mg of **27** as a white solid (0.43 mmoles, yield 84%); m.p. 194-196°C; t_R (HPLC) 10.3 min. gradient profile time (min.) 0 (B 20%), 3 (B 30%), 4 (B 50%), 12 (B 60%),

15 (B 80%); m.p. 204°C; $\upsilon_{max}(CDCl_3)/cm^{-1}$ 3456, 3395, 1713, 1668, 1501; FAB-MS m/z 406 (MH^+), 375, 298; $\delta_H(CDCl_3)$ 7.40-7.30 (5H, m), 6.90 (1H, d 7.6 Hz), 5.91 (1H, m), 5.30 (1H, m), 5.15 (2H,s), 4.38 (2H, m), 3.14 (1H, dd 4.8, 15.5 Hz), 2.80 (1H, dd 5.0, 15.5 Hz), 2.71 (3H, d 4.8 Hz), 2.69 (3H, s), 1.39 (3H, t 7.09 Hz); $[\alpha]_D^{25}$ = -18.8° (c 0.6, $CDCl_3$) *Found*: C, 56.98%, H, 5.96, N, 9.71, *calculated for* $C_{19}H_{23}N_3O_5S$: C, 56.28%, H, 5.71 N, 10.36; chiral HPLC: t_R 11.90 min. mobile phase B 30%, flow 1 ml/min.

Compound **28**. - 0.44 ml of sodium hydroxide 1N (0.44 mmoles) were added to a solution of 90 mg of **27** (0.22 mmoles) in 2.5 ml of dioxane/water (4:1). After 5 h. the solution was chilled at 0°C, diluted with water and acidified with hydrochloric acid to pH=1. The precipitated was filtered, washed with water and dissolved in chloroform (10 ml). The organic phase was dried on sodium sulfate and concentrated *in vacuo* yielding 40 mg of the corresponding acid (0.106 mmol) that was condensed with benzylamine (0.034 ml, 0.318 mmol) following the same procedure described for **27**. Purification by flash chromatograpy on silica gel ($CHCl_3$/MeOH 98:2) gave 30 mg of **28** (0.066 mmoles, yield 30%); t_R (HPLC) 11.7 min. gradient profile as for **27**; $\upsilon_{max}(CDCl_3)/cm^{-1}$ 3460, 3412, 1718, 1666, 1504; FAB-MS m/z 467 (MH^+, 100%); $\delta_H(CDCl_3)$ 7.56 (1H, b.s.), 7.35 (10H, m), 6.81 (1H, b.s.), 5.45 (1H, m), 5.25 (1H, m), 5.14 (2H, s), 4.59 (2H, d 6.0 Hz), 2.95 (1H, m), 2.80 (3H, s), 2.79 (1H, m), 2.67 (3H, d 4.85 Hz); $[\alpha]_D^{25}$ = -17.9° (c 0.4, $CHCl_3$); chiral HPLC : t_R 12.91 min. mobile phase B 30%, flow 1 ml/min.

Compound **29**. - A solution of 11.1 mg of **23** (0.032 mmoles), 0.013 ml of triethylamine (0.09 mmoles) and 20.1 mg of Py-Bop (0.038 mmoles) in 0.5 ml of dimethylformamide was stirred at R.T. for 15 min. To this solution deprotected **28**[21] (12 mg, 0.026 mmoles) was added and the reaction stirred for 1 h. The reaction mixture was diluted with sodium hydroxide 0.1N (5 ml) and extracted with ethyl acetate (2x 4 ml). The crude material obtained after drying with sodium sulfate and concentration *in vacuo* was treated for 5 min. with 0.5 ml of trifluoroacetic acid. The trifluoroacetic acid was removed in vacuo and the residue was rected at R.T. for 2 h in a mixture of 0.3 ml of pyridine and 0.025 ml of acetic anhydride. The reaction was diluted with methylene chloride (5 mL) and washed with hydrochloric acid 1N (5 ml) and water (5 ml). Purification on silica gel ($CHCl_3$/MeOH 95:5) gave 9 mg of **29** (0.015 mmoles, yield 57.6%); t_R (HPLC) 9.43 min. gradient profile time (min.) 0 (B 30%), 5 (B 50%), 12 (B 50%), 13 (B 80%); with the same gradient profile compound **12b** has a t_R 9.63 min; FAB-MS m/z 601 (MH^+, 100%), 569 (9%); ^1H and ^{13}C NMR see Table II; $[\alpha]_D^{25}$ = -46.3° (c 0.06, $CDCl_3$); chiral HPLC : t_R 12.91 mobile phase B 30%, flow 1.5 ml/min.

Compound **30**. - 40 mg of **27** (0.098 mmoles) and 0.58 ml of thioanisole were stirred in 2 ml of trifluoroacetic acid for 22 h at R.T. After evaporation, the residue was dissolved in 2 ml of pyridine, 0.3 ml of acetic anhydride and stirred overnight. The mixture was diluted with chloroform (10 mL), washed with ice-cold hydrochloric acid 1N (10 ml), water (10 ml) and the organic phase dried on sodium sulfate and concentrated *in vacuo*. Purification by flash chromatography on silica gel ($CHCl_3$/MeOH 98:2) gave 23 mg of **30** (0.073 mmoles, yield 74.4%) as a white solid ; m.p. 232-234°C; t_R (HPLC) 4.86 min gradient profile time(min.) 0 (B 20%), 3 (B 30%), 4 (B 50%), 12 (B 60%), 15 (B 80%); υ_{max}(nujol)/cm^{-1} 3312, 3260, 1705, 1645; FAB-MS m/z 314 (MH^+, 100%); $\delta_H(CDCl_3)$ 7.78 (1H, d 7.9 Hz), 5.99 (1H, m), 5.53 (1H, m), 4.39 (2H, m), 3.17 (1H, dd 4.2, 15.0 Hz), 2.74 (3H, d 4.9 Hz), 2.72 (2H, m), 2.69 (3H, s), 2.09 (3H, s) 1.40 (3H, t 7.1 Hz); $[\alpha]_D^{25}$ = -51.45° (c 0.9, $CHCl_3$-MeOH 1:1); *Found*: C, 49.82%, H, 6.13, N, 13.22, *calculated for* $C_{13}H_{19}N_3O_4S$: C, 49.8%, H, 6.07 N, 13.4;

Compound **31**. - A solution of 74 mg of **26** (0.21 mmol), 0.09 ml of triethylamine (0.64 mmoles) and 134 mg of benzotriazol-1-yl-oxy-tripyrrolidinophosphonium hexafluorophosphate (Py-Bop) (0.25 mmoles) in 2 mL of DMF

was stirred for 15 min. Benzylamine (0.047 mL ; 0.43 mmoles) was added and the reaction stirred for 1 h at R.T. The reaction mixture was diluted with sodium hydroxide 0.1 N (5 ml) and extracted with ethyl acetate (2 x 5 ml). The organic phase was washed with water (10 ml), dried on sodium sulfate and concentrated in vacuo. The residue was purified on silica gel (hexane/ethyl acetate 3:1) to give 55.2 mg of **31** (0.12 mmoles, yield 57%); t_R (HPLC) 17.7 min gradient profile time (min.) 0 (B 20%), 3 (B 30%), 4 (B 50%), 12 (B 60%), 15 (B 80%); $\upsilon_{max}(CDCl_3)$/ cm^{-1} 34, 1713, 1663, 1504; δ_H ($CDCl_3$) 7.68 (1H, b.s.), 7.40-7.28 (5H, m), 5.10 (2H, q), 4.80 (1H, m), 4.60 (2H, d 6.0 Hz), 3.49 (3H, s), 2.23 (1H, m), 1.44 (9H, s), 0.97 (3H, d 6.7 Hz), 0.90 (3H, d 6.7 Hz); $[\alpha]_D^{25}$ = -29.8° (c 0.3, $CHCl_3$).

Compound **32**. - To a suspension of 80 mg of **30** (0.25 mmoles) in 6 ml of dioxane was added 1.02 ml of sodium hydroxide 1N (1.02 mmoles). After stirring for 3 h at R.T., the solution was brought to pH= 7, concentrated *in vacuo* and finally diluted with water. The suspension was then acidified to pH= 2, the precipitated filtered and washed with water. The solid material was dried for 4 h at 90°C *in vacuo* to give 60 mg of **32** (0.21 mmoles, yield 84%) as white solid; t_R (HPLC) 1.50 min. gradient profile as for compound **30**; υ_{max}(nujol)/cm^{-1} 3290, 2679-1900, 1715, 1657, 1624, 1541, 1499; δ_H (DMSO-d_6) 12.7 (1H, b.s), 8.56 (1H, d 7.5 Hz), 7.84 (1H, b.s.), 5.35 (1H, m), 2.83 (1H, dd 5.8, 15.3 Hz), 2.63 (3H, s), 2.59 (1H, m), 2.55 (3H, d 3.8 Hz), 1.85 (3H, s); $[\alpha]_D^{25}$ = -59.4° (c 0.25, DMSO-d_6); CI/MS 286 (MH^+, 90%), 229 (38), 158 (100), 144 (40).

Compound **33**. - 14 mg of **32** (0.049 mmol), 0.025 ml of triethylamine (0.14 mmol) and 30.6 mg of Py-Bop (0.058 mmol) in 1.5 mL of DMF were stirred for 15 min. at room temperature. 27.5 mg of **31** (0.06 mmol) were deprotected by trifluoroacetic acid[21], the crude material dissolved in 0.5 mL DMF and then added to the reaction. After stirring for 2 h., the mixture was diluted with 10 mL of NaOH 0.1 N and extracted with ethyl acetate (3x5 ml). The organic phases were washed with water (10 ml), dried on sodium sulfate. After concentration in vacuo the residue was purified on silica gel ($CHCl_3$/MeOH 95:5) to give 20 mg of **33** (0.033 mmoles, yield 67.3%); $\upsilon_{max}(CDCl_3)$/cm^{-1} 3408, 2966, 2930, 1666, 1549, 1506; t_R 12.8 min. mobile phase B 30%; FAB-MS m/z 601 (MH^+ , 50%), 569 (10), 460 (25), 308 (100); δ_H ($CDCl_3$) 7.95-7.89 (3H, m), 7.39-7.26 (5H, m), 5.88 (1H, m), 5.50 (1H,m), 5.22 (1H, dd 5.6, 9.2 Hz), 5.07 (2H, q), 4.62 (2H, dq), 3.47 (3H, s), 2.90 (1H, dd 4.4, 15.3 Hz), 2.73 (3H, s), 2.72 (1H, m), 2.67 (3H, d), 2.39 (1H. m), 2.05 (3H, s), 1.0 (3H, d 6.8 Hz), 0.98 (3H, d 6.8 Hz); $[\alpha]_D^{25}$ = -27.0° (c 0.20, $CDCl_3$); *Found*: C, 55.80%, H, 5.79, N, 13.4, *calculated for* $C_{29}H_{36}N_6O_5S_2$: C, 56.0%, H, 6.0, N, 14.0.

Hydrolysis of compound **1** (MDL 62,879). - Compound 1 was treated in hydrochloric acid 18.5% for two hours at 100°-120°C. The reaction mixture was cooled, diluted with water, taken to pH=3.3 and left at 4°C over night. The precipitate was filtered (EKS-supra) and the filtrate analyzed by HPLC. Most of the peaks showed an UV spectrum indicating the presence of the thiazole ring. Purification was performed on a S 112 column using a gradient of MeOH (0 to 50%) in water. Two fractions were collected according to their HPLC profile, lyophilized and analyzed by mass spectroscopy. The FAB/MS and MS/MS measurements were performed on samples dissolved in thioglycerol immediately before the analysis. Positive daughter ions of the protonated molecular ions were recorded using the second quadrupole as collision cell (collision gas Ar ; collision energy 20 eV).
HPLC analysis : Column Ultrasphere ODS (Beckman, 25 cm x 4.6 cm), mobile phase A: ammonium formate 0.08 M / acetonitrile (95:5 v/v), mobile phase B : ammonium formate 0.08 M /acetonitrile / tetrahydrofurane (2:4:4 v/v), flow 1.5 ml/min, UV 250 nm.

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