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Antibiotics GE23077, Novel Inhibitors of Bacterial RNA Polymerase

II. Structure Elucidation

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Abstract During the screening program for new antibacterial agents produced by actinomycetes, GE23077 was isolated from fermentation broths of an *Actinomadura* sp. strain as a complex of factors A1, A2, B1, B2.

NMR, MS and GC/MS analysis carried out on the isolated components led to the conclusion that GE23077 is a novel cyclic heptapeptide consisting of common and unusual amino acids. The chemical structures of the complex components were elucidated.

Components A and B differ in the structure of the acyl group connected to a 2,3-diaminopropanoic acid moiety. A α-amino-malonic acid residue in the peptidic sequence is the origin of an isomerization process between A1 and A2 as well as B1 and B2. The chirality of the α-amino-malonic acid residue can be inverted easily *via* keto-enol tautomerism. Factors A2 and B2 should be considered as epimers of A1 and B1 respectively.

By degradation studies the absolute configuration of some amino acids were determined. Chiral GC-MS and Micellar Electrokinetic Capillary Chromatography (MEKC) were used to define the absolute stereochemistries of five out of ten chiral centers.

Keywords cyclic heptapeptide, RNA polymerase inhibitor

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Introduction

GE23077 complex, produced by *Actinomadura* sp. is a potent inhibitor of *E. coli* rifamycin sensitive (rifa-s) and resistant (rifa-r) RNA polymerase [1]. The structure has been determined by physico-chemical methods applied to the intact molecules and to their main hydrolysis products.

GE23077 complex is a mixture of four major factors, A1, A2, B1 and B2, having molecular weights of 803 (A1, A2) and 805 Da (B1, B2).

In this paper we describe the structural determination of the natural complex through spectroscopic and chromatographic methods. Homo- and heteronuclear 2D NMR spectroscopy indicated that all the factors are cyclic heptapeptides containing three natural and four unusual amino acids. The correct sequence was obtained by heteronuclear multiple-bond connectivity (HMBC) [2] and was confirmed by MS studies on the fragments obtained from a complete and partial hydrolysis.

The factors of GE23077 differ only in the residue 2,3-diamino-propanoic acid where the 3-amino group forms an amide with aliphatic or olefinic acid. The peptides contain an amino malonic acid, which is the origin of an isomerization process between A1 and A2 as well as B1 and B2. Due to the acidic character of the α proton of this amino acid the two possible epimers occur in protic solvents (keto-enol tautomerism).

The absolute configurations of some amino acids constituting GE23077 were defined by applying chiral GC/MS and Micellar Electrokinetic Capillary Chromatography (MEKC) to a suitably derivatized hydrolysate of the natural compound.

Results and Discussion

The four main factors, A1, A2, B1 and B2 of GE23077 were separated by preparative HPLC.

During the isolation of individual peaks, an equilibration between factors 1 and 2 (A and B) was observed. In particular the purified A1 (as sodium or ammonium salt) equilibrates to a mixture of A1 and A2, as well as A2 equilibrates to a mixture of A2 and A1 in about 5 hours. The same behaviour was noticed for B1 and B2. The time of equilibration (to 1 : 1) in aqueous medium was studied by HPLC and shown to be pH and temperature dependent.

The LC-ESI/MS analysis of the complex showed that the molecular weights of the individual components were 803 for A1 and A2 and 805 for B1 and B2. The elemental analysis carried out on the factors A and B separately resulted: C 46.52%, H 6.28%, N 15.63% and C 46.48%, H 6.32%, N 15.60% respectively in very good agreement with the calculated values for $C_{31}H_{49}N_9O_{16}$ and $C_{31}H_{51}N_9O_{16}$. The FAB spectra of the isolated peaks A1 and B1 showed a very similar fragmentation pattern, but with a mass shift of +2 u ($[MH]^+$ *m/z* 806; $[M-H]^-$: *m/z* 804) for B1. This suggests that factor B1 has a similar structure as factor A1 with a different degree of unsaturation.

The positive FAB spectrum of factor A1 reveals that the lowest isotope of the main peak has an atomic mass of 782 u, which is due to the loss of CO_2 from the molecule (m.w. 803) and the consequent addition of Na^+. This is strongly supported by the presence of the peaks at *m/z* 804 ($[M+H]^+$) and at *m/z* 826 ($[M+Na]^+$). The isotope pattern of the protonated species indicates that besides C, H, N and O no elements containing higher mass isotopes are present. Furthermore, no typical fragments corresponding to the loss of amino acid residues are present and this indicates that the compound is not a linear peptide.

Structure Elucidation by NMR

The structures of the main components of the complex have been determined by various homo- and heteronuclear 2D-experiments. During NMR studies, starting from the purified factors A1 or A2, a mixture of both isomers in an approximate ratio 1 : 1 was obtained in 12 hours in methanol-d_4 at 2°C. Because of the rapid equilibration in protic solvents, the single component isolation required special care. In aprotic solvents only one of the two isomers seemed to be stable. In DMSO-d_6 A2 was completely converted into factor A1 in 12 hours at room temperature; under the same conditions factor B2 gave exclusively factor B1.

Therefore the structure elucidation by NMR was carried out in this solvent to circumvent problems of signal overlap and to achieve higher concentrations.

The ^1H spectra of GE23077-A1 and B1 are very similar and reflect a pattern that is typical for small cyclic peptides.

Factor B1

In the ^1H NMR spectrum of GE23077 factor B1 seven signals corresponding to amide protons are observed between 7.6 and 9.0 ppm. A further amidic NH was found at 7.30 ppm. Presaturation of the H_2O signal reveals the presence of five hydroxyl groups in the range between 4.8 and 6.0 ppm which must be connected to aliphatic carbons.

The structure of factor B1 was elucidated by several NMR techniques such as DQF-COSY [3], TOCSY [4], ROESY [5], HMQC and HMBC. DQF-COSY and TOCSY spectra revealed the intra-residual spin systems of 7 different amino acids including valine (Val), threonine (Thr), serine (Ser) and other unusual amino acids.

The amide proton at 7.93 ppm is connected to a CH_2 unit followed by a CH(OH) fragment forming an iso-serine (IsoSer) moiety.

The amide proton at 8.02 ppm is coupled with a CH fragment, which does not show any further coupling to other protons. This system is referred to Bbb. Two amide protons at 8.94 and 7.82 ppm, connected each other *via* $CH–CH_2$ fragment, reveal the spin system of the uncommon amino acid residue named Ccc. The DQFCOSY spectrum shows that the amide proton at higher field is bound to the CH_2 unit. Ccc corresponds to 2,3-diaminopropanoic acid, a residue found in the antibiotic edeine [6]. Analysis of the homonuclear 2D spectra enables us to identify also the amino acid β,γ-dihydroxyglutamine (Dhg) previously isolated from a plant [7]. The primary amide proton resonances at 7.32~7.26 ppm were assigned by ROESY and HMBC spectral analysis. In the HMBC spectrum a carbonyl resonance at 175.5 ppm shows a coupling to the primary amide signal and to CH and OH protons in position γ of "Dhg".

Examining further correlations in the HMBC spectra the sequence of the cyclic peptide was established as *cyclo*(Ser-Val-Dhg-Thr-IsoSer-Bbb-Ccc-) as reported in Fig. 1.

The linkage between Ser and Ccc was attested by the long-range correlations observed for the Ccc carbonyl resonance at 169.0 ppm that shows a strong coupling to the α-proton of Ser residue at 4.47 ppm. Other long-range correlations are shown in Fig. 1.

HMBC and homonuclear 2D spectra analysis allowed the assignment of the acyl group referenced as Yyy as well. The carbonyl resonance at 172.3 ppm in fact shows strong coupling to the amide proton at 7.82 ppm of 2,3-diaminopropanoic acid (Ccc) and to a signal at 1.95 ppm.

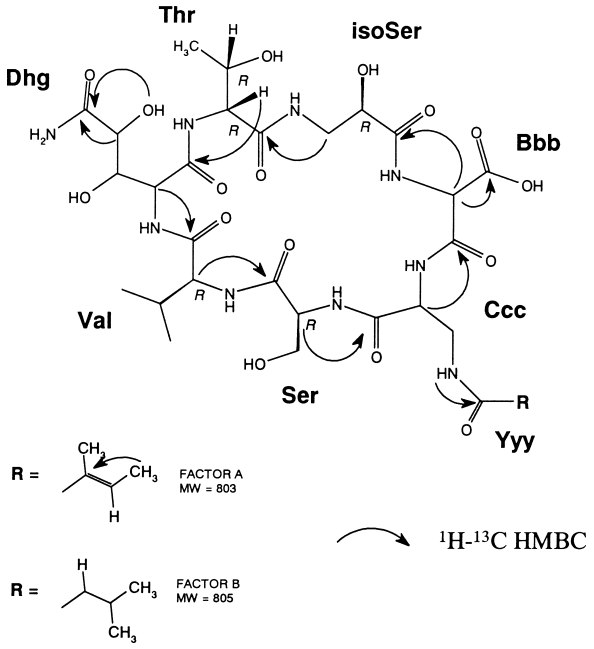


Fig. 1 Structures of GE23077-A1 and B1.

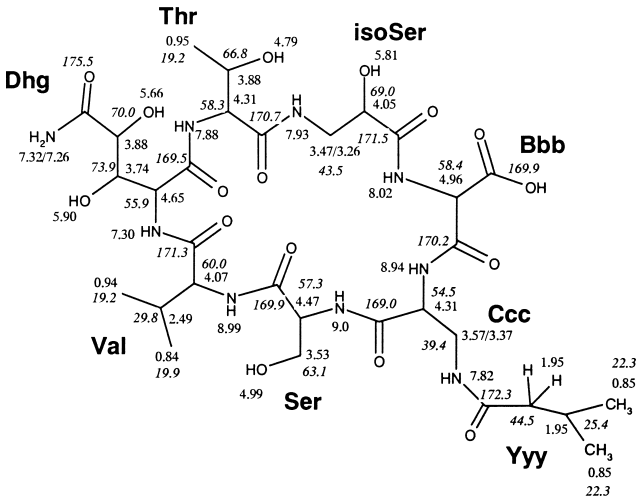


Fig. 2 Structure of GE23077-B1.

The integration of the resonance at 1.95 ppm is consistent with three protons and at least one of them experiences homonuclear scalar coupling with two methyl groups at 0.85 ppm. To clarify this spin system a DEPT-HMQC [8] experiment was acquired. This proton carbon correlation spectrum establishes that at 1.95 ppm the overlapping of CH_2 (δ_C: 44.5 ppm) and CH (δ_C: 25.4 ppm) resonances occurs. For factor B1 the complete Yyy spin system could be determined as that of isobutyric acid forming an amide bond with the 3-amino function of 2,3-diaminopropanoic acid (Ccc). In Fig. 2 the structure of B1 factor with the correspondent ^{1}H and ^{13}C chemical shifts is reported.

Factor A1

GE23077-A1 NMR spectra are very similar to the spectra obtained for factor B1. Except some small changes in chemical shifts, the only significant difference is found in the acyl group Yyy. In A1, the proton spectrum contains an olefinic signal at 6.34 ppm that experiences a strong homonuclear scalar coupling to a methyl group at 1.70 ppm and a weak coupling to a second methyl group at 1.72 ppm. A standard HMBC experiment shows that both methyl groups and the olefinic proton are correlated with a quaternary carbon at 131.6 ppm. In the selective HMBC [9] spectrum the olefinic proton at 6.34 ppm and the methyl group at 1.72 ppm exhibit also a strong coupling to the carbonyl carbon at 168.8 ppm. This carbonyl resonance also experiences coupling to the β-amide proton of Ccc at 7.67 ppm. The structure of residue Ccc-Yyy for factor A1 is therefore described as 2,3-diaminopropanoic acid where the 3-amino group forms an amide with 2-methyl-2-butenoic acid. The *E*-geometry of the double bond in Yyy was shown in ROESY spectrum by two NOE cross-peaks of the proton at 6.34 ppm with the Ccc protons at 7.67 ppm (β-NH) and 3.59 ppm (β-CH).

Table 1 contains proton and carbon chemical shifts assignments of GE23077-A1 and GE23077-B1.

The Mechanism of Isomerization

The structures of GE23077-A1 and GE23077-B1 contain several unusual fragments that could be the origin of the isomerism.

The α-aminomalonic acid (Bbb) contains a very acidic α proton due to the two carboxylic groups. Therefore equilibrium between *R* and *S* epimer *via* an enolic transition state seemed to be possible. In fact this keto-enol tautomerism could be demonstrated by further NMR experiments in D_2O where the α proton disappeared after several days due to exchange with the solvent.

MS Analysis of Acid Hydrolysates of the GE23077 Complex

The acidic hydrolysis carried out as described in the experimental section gave a residue that, after derivatization, was analyzed by GC-MS. This analysis confirmed the presence of the natural amino acids valine, serine, threonine and glycine (derived from the decarboxylation of the α-aminomalonic acid) and a number of non-natural amino acids.

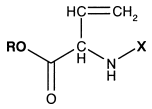
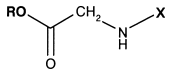
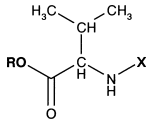
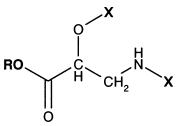
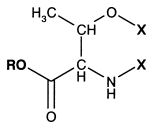
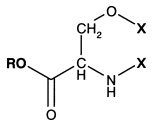
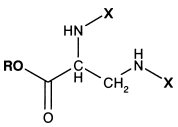
While the natural amino acids were identified by comparison with standards, the structures of two non-natural amino acids (iso-serine and 2,3-diamino propanoic

Table 1 NMR assignment of GE23077-A1 and B1[a)]

		Factor A1		Factor B1	
		δ_H (DMSO-d_6)	δ_C (DMSO-d_6)	δ_H (DMSO-d_6)	δ_C (DMSO-d_6)
Ser	NH	8.93	—	9.0	—
	α	4.47	57.3	4.47	57.3
	β	3.53	63.1	3.53	63.1
	OH	5.00	—	4.99	—
	C′	—	170.0	—	169.9
Val	NH	8.94	—	8.99	—
	α	4.07	60.1	4.07	60.0
	β	2.50	29.8	2.49	29.8
	γ(1)	0.94	19.3	0.94	19.2
	γ(2)	0.85	19.9	0.84	19.9
	C′	—	171.3	—	171.3
Dhg	NH	7.29	—	7.30	—
	α	4.65	55.9	4.65	55.9
	β	3.74	74.0	3.74	73.9
	γ	3.88	69.9	3.88	70.0
	β-OH	5.97	—	5.90	—
	γ-OH	5.68	—	5.66	—
	NH_2	7.33/7.27	—	7.32/7.26	—
	C′	—	169.9	—	169.5
	γ-C′	—	175.6	—	175.5
Thr	NH	7.88	—	7.88	—
	α	4.31	58.2	4.31	58.3
	β	3.90	66.8	3.88	66.8
	γ	0.96	19.2	0.95	19.2
	OH	4.82	—	4.79	—
	C′	—	170.7	—	170.7
IsoSer	NH	7.93	—	7.93	—
	α	4.05	69.1	4.05	69.0
	β	3.42/3.25	43.4	3.47/3.23	43.5
	OH	5.79	—	5.81	—
	C′	—	171.5	—	171.5
Bbb	NH	8.04	—	8.02	—
	α	4.94	58.5	4.96	58.4
	C′	—	170.0	—	169.9
	β-C′	—	170.2	—	170.2
Ccc	NH(1)	8.93	—	8.94	—
	NH(2)	7.67	—	7.82	—
	α	4.37	54.3	4.31	54.5
	β	3.59/3.49	39.7	3.57/3.37	39.4
	C′	—	169.0	—	169.0
Yyy	C′	—	168.8	—	172.3
	α	—	131.6	1.95	44.5
	β	6.34	129.9	1.95	25.4
	β-CH_3	1.70	12.3	0.85	22.3
	α-CH_3	1.72	13.7	—	—

[a)] Referenced to DMSO at 2.50 and 39.5 ppm respectively

Table 2 Structure assignments of the relevant GC-MS peaks

Peak n°	R=C_4H_9 X= COCF3	Rt (minutes)	PICI
1		7.49	254: $[MH]^+$ 197: $[MH-C_4H_9]^+$
2 Gly		8.0	228: $[MH]^+$ 172: $[MH-C_4H_8]^+$
3 Val		10.10	270: $[MH]^+$ 197: $[MH-C_4H_9O]^+$ 169: $[MH-C_5H_9O_2]^+$
4 IsoSer		10.29	354: $[MH]^+$ 298: $[MH-C_4H_8]^+$ 241: $[MH-CF_3CONH_2]^+$
5 Thr		10.32	368: [MH]+ 312: $[MH-C_4H_8]^+$
6 Ser		10.48	258: $[MH]^+$ 202: $[MH-C_4H_8]^+$ 157: $[MH-C_5H_9O_2]^+$
7 Ccc		13.28	353: $[MH]^+$ 296: $[MH-C_4H_9]^+$ 227: $[MH-C_4H_9CF_3]^+$

acid) were derived from the interpretation of the EI and PICI spectra. The most relevant data together with the interpretation are reported in Table 2. In addition, "controlled" hydrolysis (quenching after 15, 30, 60 and 90 minutes) with subsequent FAB-MS analysis confirmed the sequence proposed by NMR. In Table 3 the structure assignments of main FAB/MS ions are reported.

Chirality of Some Amino Acids

The stereochemistry of some amino acids could be determined both by chiral GC-MS and by application of MEKC according to Nishi's method [10].

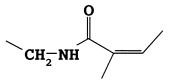
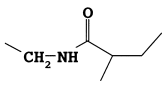
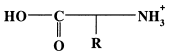
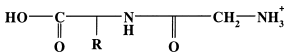
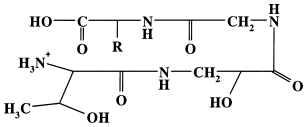
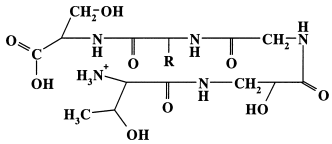
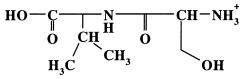
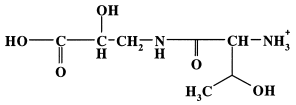
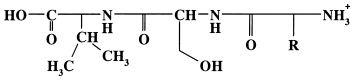
For the gas chromatographic chiral separation acid-hydrolyzed products of GE23077 were derivatized with *n*-butanol (*n*-BuOH) and trifluoroacetic anhydride (TFAA) while in MEKC analysis 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (TAGIT) was selected as a pre-column chiral derivatizing agent. By comparing the "natural" amino acids contained in GE23077 hydrolysate with authentic standard samples, the chiralities of Val, Ser, allo-Thr were assigned to the D-configuration.

The chiralities of the unnatural amino acids could not be determined unambiguously due to the absence of reference compounds or degradation/epimerisation during the hydrolysis. In the case of isoserine, the D chirality was assigned on the assumption that the elution order is the same as for the common amino acids.

Conclusion

Based on NMR, MS and GC/MS data the structure of the natural complex GE23077 was determined. Each factor is a cyclic heptapeptide characterized by the sequence: D-Ser-D-Val-Dhg-D-allo-Thr-D-IsoSer-Bbb-Ccc (with Bbb=α-aminomalonic acid, Ccc=2,3-diaminopropanoic acid). Components A and B differ in the structure of the acyl group Yyy which is connected to the β-amino group of the 2,3-diaminopropanoic acid moiety (Ccc). The chirality of the α-aminomalonic acid residue (Bbb) can be inverted

Table 3 Structure of the main peaks from the "controlled" hydrolysis

[MH]$^+$	R =  Factor A	and	R =  Factor B
187/189			
244/246			
432/434			
519/521			
205			
207			
373/375			

easily *via* keto-enol tautomerism. Therefore, factors A2 and B2 should be considered as epimers of A1 and B1 respectively.

The absolute stereochemistry of five out of ten chiral centers was defined by chiral GC-MS and MEKC.

Experimental

The samples used for the structure elucidation were obtained as sodium or ammonium salts of individual factors by preparative HPLC.

The HPLC analyses were carried out on reverse phase column (Beckman ultrasphere ODS C-18, 5 μm, 4.6×250 mm) using 3 : 50 mixture of acetonitrile/water with ammonium formate (0.04 M, pH 7) as eluting solvent in isocratic mode. For detection, UV operating at 230 nm was used.

Chemicals

The following standard amino acids of analytical grade were used: D,L-2,3-diaminopropionic acid (DAPA) mono hydrochloride, D,L-Isoserine (Fluka, Buchs, Switzerland), L-Valine (Janssen, Beerse, Belgium), Glycine (Merk, Darmstadts, Germany), D-Serine, D-Allo-Threonine (SIGMA, St. Louis, MO, USA). 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (TAGIT) was purchased from Fluka (Buchs, Switzerland). Sodium dodecyl sulfate (SDS) was supplied from Sigma (St. Louis, MO, USA). All the solvents used were of HPLC grade while the salts were reagents of analytical grade.

NMR Spectroscopy

All the NMR spectra were recorded on a Bruker AMX 600 using 8 mmolar sample of peptide in DMSO-d_6 at 300 K. Spectra were referenced to residual solvent signals with resonances at 2.5 (δ_H) and 39.5 ppm (δ_C).

ROESY experiments were acquired with mixing times of 150 or 300 ms. The assignment of the carbon resonances was carried out on the basis of HMQC, DEPT-HMQC and HMBC spectra. For HMQC and DEPT-HMQC spectra a BIRD pulse [11] was applied to suppress magnetization of protons connected to ^{12}C (recovery delay of 200 ms). The editing pulse in the DEPT experiment was set to 180 degrees resulting in positive cross-peaks for CH and CH_3 groups and negative cross peaks for CH_2 groups (or vice versa).

The selective HMBC spectrum was recorded with a 270 degrees Gaussian pulse (pulse length 2 ms) and a 80 ms delay for the development of long-range couplings.

Acidic Hydrolysis and Derivatization for GC-MS Analysis

The acidic hydrolysis was carried out on *ca.* 3 mg of GE23077 complex at 110°C overnight with HCl 10%/phenol 1%, in nitrogen atmosphere using a PicoTag apparatus (Waters). After cooling the reaction mixture was lyophilized in dry-seal vacuum centrifuge. Amino acids residues of GE23077 were then derivatized to obtain volatile chiral amino acids.

GE23077 hydrolysate was treated with 200 μl of *n*-butanol/HCl (2.4 N) at 100°C for 30 minutes. The butyl esters obtained were dried in a dry-seal vacuum centrifuge operating at room temperature. Finally the dry residue was acylated with 200 μl of TFAA/methylene chloride in ratio 1 : 1 keeping the vial, tightly capped, at 80°C for 10 minutes. After cooling to room temperature, excess reagents and solvent were removed under a gentle stream of nitrogen. The derivatized residue was dissolved in 100 μl of methylene chloride and 0.5 μl aliquot were injected into

GC/MS for amino acids composition and stereochemical definitions

MS Spectrometry

The positive and negative ion FAB-MS spectra were obtained on a Finnigan MAT TSQ 700 triple stage quadrupole mass spectrometer, equipped with a saddle field atom gun operating at 8 kV with Xe gas. The instrument was previously calibrated both in negative and positive mode using CsI. Prior to analysis, the samples were dissolved in DMSO and glycerol/water (1 : 1) was added as an ionization matrix.

GC/MS analysis were carried out on a Finnigan MAT TSQ700 triple quadrupole mass spectrometer directly connected with a Varian 3400 gas chromatograph equipped with a split-splitless injector.

For amino acids analysis the GC column used was Supelco SPB1, 30 m×0.32 mm i.d.; 0.25 μm thin film; the carrier gas was helium (split vent 80 ml/minute); column pressure 8 psi; splitless mode injections. The injector and interface temperatures were 260°C. The oven temperature was set at 60°C for 1 minute and increased at 12°C/minute to 260°C.

MS-EI conditions were: source temperature 150°C and electron energy 70 eV.

MS-PICI (positive ion chemical ionisation) conditions were: source temperature 140°C; electron energy 120 eV; ionization gas: isobutane; gas lines pressure: 6×10^3 torr.

For the chiral gas chromatographic separation of the amino acids in split mode injection (injected volume 0.5 μl) the column Alltech Chirasil-Val 25 m×0.25 mm, 0.2 μm thin film was used. Helium was selected as carrier gas (column pressure 10 psi, split vent 80 ml/minute); the injector and transfer line temperatures were set to 240°C and 210°C respectively. The oven temperature was set at 80°C and increased at 200°C in 24 minutes.

MS conditions were: Ion source temperature 150°C; Electron energy: 70 eV (EI); Electron multiplier: 1400 V; Scan range/time: 33,650 u/0.8 seconds.

Micellar Electrokinetic Capillary Chromatography (MEKC)

All separations were carried out on the fully automated capillary electrophoresis system SpectraPHORESIS™ 1000 delivering up to ±30 kV and equipped with a programmable, high-speed, scanning, multiple-wavelength UV-Vis detector. The multitasking SpectraPHORESIS™ software CE 1000 running on a Spectra 386/25 MHz personal computer supported data acquisition, handling and reporting.

MEKC was performed at 20°C in a 440 mm (370 mm effective length)×50 μm i.d. fused silica capillary tube. The injections were performed hydrodynamically at 0.75 p.s.i. for 1 second (the amount of sample directly injected into the capillary was 1.8 nl).

The following voltage gradient profile was applied:

T (min)	0	20	25	35
V (kV)	10	10	20	20

The maximum allowed voltage was controlled by upper limiting the run current to 300 μA.

UV detection at 210 and 254 nm was used.

The background electrolyte (BGE) consisted of a solution of 20 mM $NaH_2PO_4/Na_2B_4O_7$ at pH 9 (adjusted with NaOH 5 N) and 0.3 M sodium dodecyl sulfate (SDS).

The chemical derivatization with TAGIT was carried out at room temperature (reaction time: 15 minutes) mixing TAGIT solution (20 mg/ml CH_3CN) with GE23077 hydrolysate or standard amino acids solutions. The hydrolysate sample and amino acids solutions were prepared as a mixture 1 : 1 v/v of H_2O/CH_3CN containing 0.4% w/v of triethylamine (TEA).

The absolute configurations of the "natural" amino acids contained in GE23077 hydrolysate were assessed by comparing the electropherogram of the TAGIT-derivatized hydrolysate before and after addition of TAGIT-derivatized standard amino acids.

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