Reductive Alkylation of Glycopeptide Antibiotics: Synthesis and Antibacterial Activity

ROBIN D. G. COOPER, NANCY J. SNYDER, MARK J. ZWEIFEL, MICHAEL A. STASZAK, STEPHEN C. WILKIE, THALIA I. NICAS, DEBORAH L. MULLEN, THOMAS F. BUTLER, MICHAEL J. RODRIGUEZ, BRET E. HUFF and RICHARD C. THOMPSON

Infectious Diseases Research, Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana 46285

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Reductive alkylation of the A82846 family of glycopeptide antibiotics has the potential of producing seven products. *N*-Alkylation of the disaccharide amino function can be accomplished selectively, and offers the greatest increase in antibacterial activity. Products resulting from *N*-alkylation of LY264826 (A82846B) provide the most potent derivatives as compared to other members of this class of antibiotics. Two of these derivatives, LY307599 and LY333328 are approximately 500 times more active than vancomycin against vancomycin-resistant enterococci.

The glycopeptide antibiotic vancomycin has been an important drug for the treatment of Gram-positive bacteria since being introduced 35 years ago. As a result of resistance to other antibiotics, vancomycin is now the drug of choice for the treatment of serious Gram-positive infections. In the late 1980's, however, enterococci resistant to vancomycin were encountered,[1,2] creating a situation for which there is essentially no treatment available for some of these infections. While resistance to vancomycin has not yet spread to staphylococci or streptococci in the clinic, experts agree it may be only a matter of time. The resistance to enterococci alone has nonetheless created an urgent need for the development of new therapies.

Previous reports have demonstrated that *N*-alkylation of the vancosaminyl moiety of vancomycin can result in enhanced activity,[3] and in some cases, restore the effectiveness of the glycopeptide toward vancomycin-resistant enterococci (50-fold increase in activity).[4] While this is indeed important work, these semisynthetic vancomycin derivatives lack the potency necessary for further development. Extension of this SAR to the A82846 family of natural glycopeptides, however, revealed unanticipated antimicrobial activity. Significantly, the activity of the *p*-chlorobenzyl derivative of LY264826 (A82846B), LY191145, toward vancomycin-resistant enterococci, as well as other Gram-positive organisms, indicated the therapeutic potential of *N*-alkyl glycopeptides of the A82846 family.[5]

Recently, PAVLOV and co-workers reported[6] the alkylation of the glycopeptide eremomycin,[7] which is identical to A82846A,[8] with alkyl and arylalkyl halides. These studies established that, under these reaction conditions (alkyl halide, $NaHCO_3$), substitution occurs preferentially at the *N*-methylleucine moiety, followed by alkylation of the carboxyl group to afford ester derivatives. These substitutions resulted in comparable or lower antibacterial activity against Gram-positive bacteria as compared to vancomycin and eremomycin, however all derivatives were inactive against vancomycin-resistant enterococci.

Herein we report our findings for the selective mono-alkylation of the glycopeptide antibiotic LY264826 by reductive alkylation and the application of this methodology to other structurally-related natural glycopeptides. Of significance, we found that *N*-alkylation of LY264826 on the disaccharide amino function offered the greatest advantage and presents a promising outlook for successfully negotiating the resistance mechanisms presented by the enterococci.

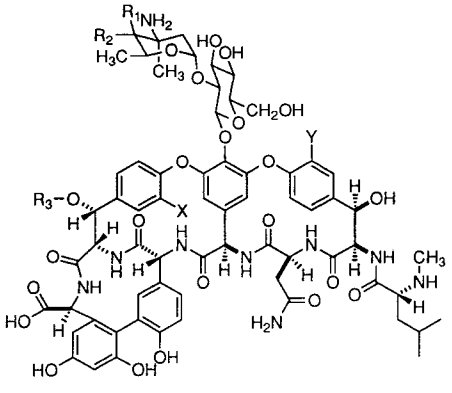
Chemistry

The A82846 family of glycopeptide antibiotics are structurally similar to vancomycin, the major difference being the presence of an additional sugar appended to the benzylic hydroxyl function of amino acid residue 6 (Fig. 1); a more subtle difference is the stereochemistry at the 4-position of the amino-sugar moiety of the disaccharide. The relationship between the three members of the A82846 family is merely the degree of chlorination of amino acid residues 2 and 6. Orienticin A,[9,10] the remaining monochloro-regioisomer, can be obtained either from natural sources, or by the selective monodechlorination of LY264826.[8] The most active

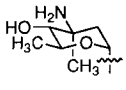
member of this class of antibiotics, LY264826, is four to eight times more potent than vancomycin and was, therefore, selected as the primary candidate for chemical derivatization (Table 1).

The reductive alkylation of LY264826 is accomplished by treating a methanolic solution of the glycopeptide with a slight excess of the desired aldehyde, heating to reflux and adding to the resulting solution a suitable reducing agent, typically sodium cyanoborohydride (Fig. 2). The progress of the reaction can best be monitored by analytical HPLC, and purification of the products is accomplished by preparative HPLC (C_{18} reverse phase).

Fig. 1. Structures of the A82846 family of antibiotics and vancomycin.



Vancomycin:
X=Cl, Y=Cl, R_1=OH, R_2=H, R_3=H
A82846A (LY264825):
X=H, Y=Cl, R_1=H, R_2=OH, R_3=4-epivancosamine
A82846B (LY264826):
X=Cl, Y=Cl, R_1=H, R_2=OH, R_3=4-epivancosamine
A82846C (LY264827):
X=H, Y=H, R_1=H, R_2=OH, R_3=4-epivancosamine
Orienticin A:
X=Cl, Y=H, R_1=H, R_2=OH, R_3=4-epivancosamine

where 4-epivancosamine= 

The three amino functions of LY264826 are all capable of undergoing reductive alkylation which can result in seven possible products—one tri-, three di- and three mono-alkylated products. Under these reaction conditions, the major product obtained is that resulting from mono-alkylation of the disaccharide amino function (N1); characteristically, this product is also the first to elute by HPLC. The minor product obtained results from mono-alkylation of the *N*-methylleucine amino function (N2). The reaction conditions can be modified, typically by employing a large excess of aldehyde and longer reaction times, to obtain sufficient quantities of the di- and tri-alkylated products. We have not been able to isolate, in pure form, the product resulting from mono-alkylation of the mono-saccharide amino function (N3).

Fast atom bombardment mass spectroscopy (FAB-MS) was utilized to determine the structures of the *N*-alkylated glycopeptides.[3,11,12] The observed molecular ion is indicative of the degree of alkylation (mono-, di-, or tri-) while the fragmentation pattern is characteristic of the site of alkylation. To illustrate this, the mass spectrum for compound **4** (LY307599) is reproduced in Fig. 3. The molecular ion cluster at *m/z* 1759 indicates a mono-alkyl derivative. The fragment at *m/z* 1143 represents the aglycone, indicating that N2 is not substituted. The fragment at *m/z* 1615 results from loss of the 4-epivancosamine sugar only, while *m/z* 1287 results from loss of the disaccharide plus the alkyl group. This fragmentation pattern is uniquely consistent with substitution at N1 (disaccharide amino function).

Results and Discussion

Our initial studies focused on determining which of the seven possible *N*-alkyl derivatives of LY264826 exhibited the most potent antimicrobial activity. Table 2 illustrates the various derivatives obtained from the reductive alkylation using *p*-chlorobenzaldehyde, 4-

Table 1. Comparison of the antimicrobial activity of the naturally-occuring glycopeptides.

Glycopeptide		MIC's Enterococci, μg/ml		MIC's Staphylococci, μg/ml		
		Resistant	Sensitive	SA 489	ST105	SE270
Vancomycin		128-1054	1-2	0.25	2	1
A82846A	LY264825	128-1054	1-2	0.5	0.5	0.25
A82846B	LY264826	64-16	0.13-0.5	≤0.063	1	0.25
A82846C	LY264827	>64	1-2	0.5	1	0.13
Orienticin A		>64	1-2	0.25	4	0.5

Fig. 2. Synthesis of the *N*-alkyl glycopeptides.

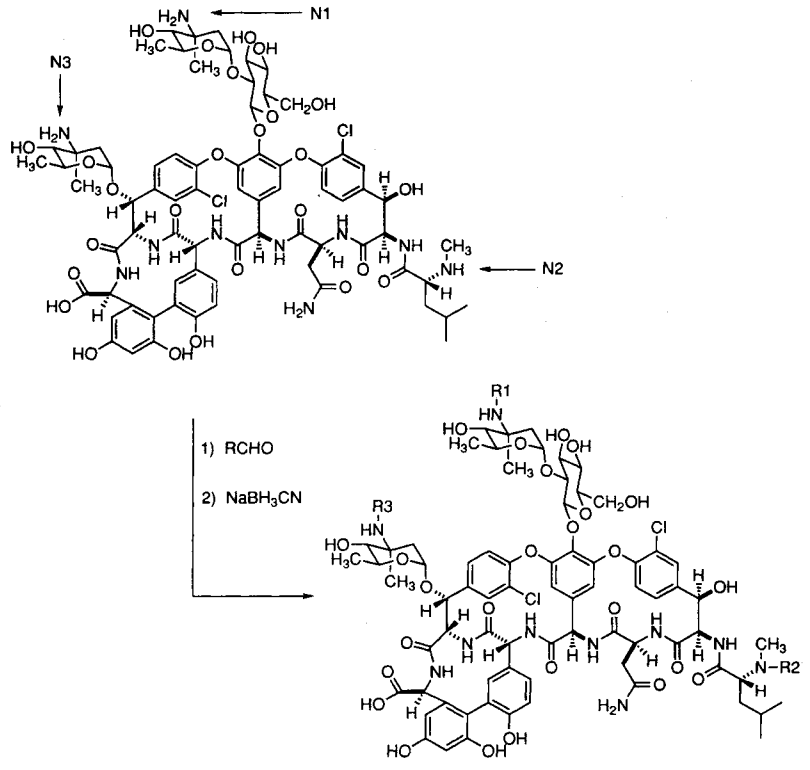
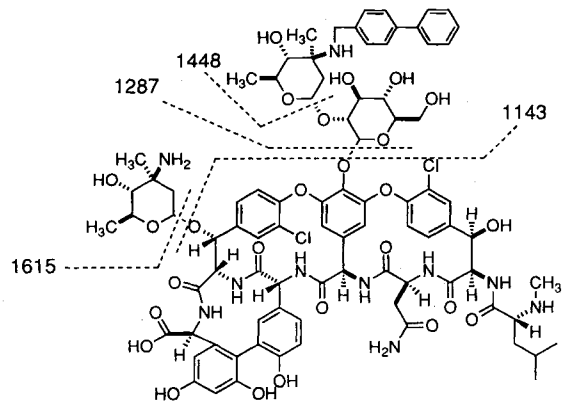
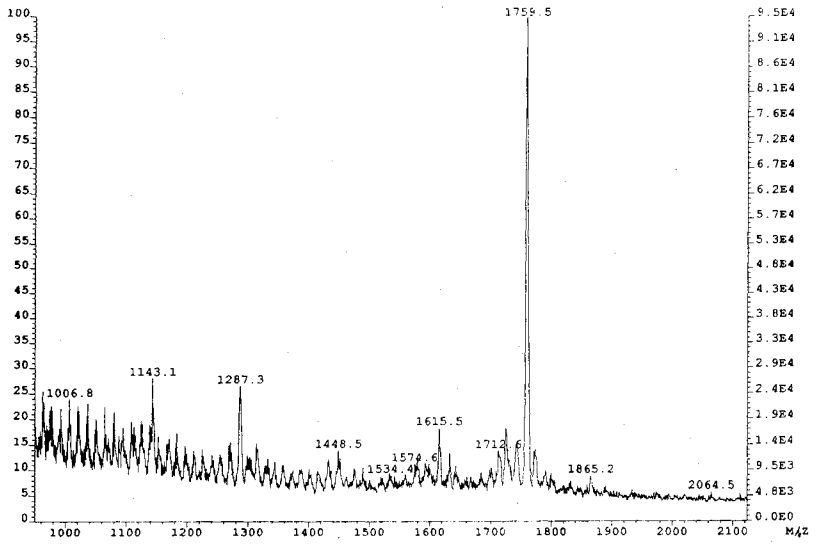


Fig. 3. FAB-MS analysis of compound **4** (LY307599).





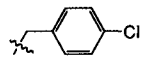
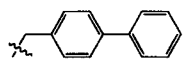
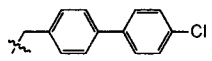
biphenylcarboxaldehyde, and 4'-chlorobiphenyl-4-carboxaldehyde.[13] The screening assay used to evaluate *in vitro* activity against enterococci consisted of four representative vancomycin-resistant strains (two *E. faecium*, and two *E. faecalis*) with confirmed *vanA* genotypes, and five vancomycin-susceptible isolates (two *E. faecium*, and three *E. faecalis*). Reported here is the range of MIC's (μg/ml) for the resistant and sensitive enterococci. The data presented for the *in vitro* evaluation of staphylococci were obtained from a single screening reference strain consisting of *Staphylococcus aureus* 489 (methicillin-resistant), *Staphylococcus epidermidis* 270, and *Staphylococcus haemolyticus* ST105.

A number of interesting trends were observed from the data. Most significant was the observation that mono-alkylation of the disaccharide amino function (N1) affords the most potent activity against enterococci (vancomycin-sensitive and resistant) and staphylococci. Two of these derivatives, **4** (LY307599) and **9** (LY333328), exhibited activity 30~100 times greater than the parent compound, LY264826, against VRE (vancomycin-resistant enterococci) while maintaining excellent activity against staphylococci. Mono-alkylation of the *N*-methylleucine moiety (N2) decreased the activity against VRE 8~16 times as compared to N1 alkylation. The di- and tri- *N*-alkyl derivatives were equally less active against enterococci than the corresponding N1 mono-alkyl products, while the tri-alkyl compounds were also completely ineffective against staphylococci. Interestingly, **7** and **12** (N1+N3 di-alkylated) were almost as active as **4** and **9**, respectively, against enterococci but were 4~30 times less active against staphylococci.

The results of this study clearly indicate that mono-alkylation of the disaccharide amino function (N1) affords the most potent compounds against enterococci and staphylococci such that subsequent SAR studies should focus on the preparation and evaluation of these derivatives. In general, the antibiotic activity of the *N*-alkyl glycopeptides follows a trend according to site(s) of substitution such that N1 > N1+N2, N1+N3 > N2 > N1+N2+N3.

Our next objective was to compare the antibiotic activity of the N1 mono-alkylated derivatives of a series of related glycopeptides. Table 3 illustrates the results from the reductive alkylation of the natural glycopeptides with 4-biphenylcarboxaldehyde and 4-butyloxybenzaldehyde. Evaluation of the antibacterial activity of these derivatives provides insights as to the role of the chlorine substituents and the monosaccharide substituted at amino acid residue 6.[14,15]

Table 2. N-alkylation of LY264826 (A82846B): Antimicrobial activity versus degree and site of alkylation.

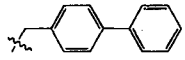
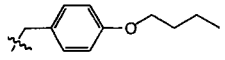
Compound	Site of Alkylation[a]	FAB-MS[b] (m/z)	R_t[c] (min)	MIC's Enterococci, μg/ml		MIC's Staphylococci, μg/ml		
				resistant	sensitive	SA 489	ST105	SE270
Sidechain = 								
1	N1	1717.4	10.8	4-16	0.13-0.25	≤0.063	0.5	0.5
2	N2	1717.8	12.7	16-64	0.5-1	0.25	4	0.5
3	N1+N2	1841.5	15.2	32->128	1-2	0.5	4	4
Sidechain = 								
4	N1	1759.5	14.3	0.5-2	0.031-0.13	0.13	1	1
5	N2	1758.6	15.0	8-32	0.25-0.5	≤0.063	4	0.25
6	N1+N2	1924.7	19.1	1-4	0.25-1	≤0.063	4	0.25
7	N1+N3	1924.6	17.4	0.5-2	0.063-0.25	4	4	0.5
8	N1+N2+N3	2090.7	22.1	4-32	0.063-0.5	>64	>64	>64
Sidechain = 								
9	N1	1793.3	15.6	0.5-2	0.031-0.13	0.5	0.5	0.13
10	N2	1793.1	16.4	4-16	0.063-0.25	≤0.063	0.5	0.13
11	N1+N2	1991.5	21.0	8-4	1-8	16	16	8
12	N1+N3	1993.9	19.8	2	0.031-2	16	16	8
13	N1+N2+N3	2194.3	24.9	>64	>32	>64	>64	>64

[a] N1 refers to the disaccharide amino function. N2 refers to the *N*-methylleucine amino function. N3 refers to the monosaccharide amino function.

[b] Reported is the base peak (typically M+2 or M+3).[12]

[c] Analytical HPLC analysis of purified product, retention time reported in minutes.

Table 3. Antimicrobial activity of *N*-alkylated glycopeptides.

Compound	Parent compound	FAB-MS[a] (m/z)	R_t[b] (min)	MIC's Enterococci, μg/ml		MIC's Staphylococci, μg/ml		
				Resistant	Sensitive	SA 489	ST105	SE270
Sidechain = 								
14	Vancomycin	1616.7	13.8	8	0.25-4	≤0.063	0.25	0.125
15	A82846A	1724.6	14.5	8-16	0.13-0.5	2	2	0.25
4	A82846B	1759.5	14.3	0.5-2	0.031-0.13	0.13	1	1
16	A82846C	1690.6	13.2	16-64	0.25-1	≤0.063	≤0.063	≤0.063
17	Orienticin A	1724.8	13.1	2-8	0.031-0.13	≤0.063	≤0.063	≤0.063
Sidechain = 								
18	Vancomycin	1612.1	14.0	16-64	0.5	≤0.063	0.25	≤0.063
19	A82846B	1755.8	14.0	0.5-1	0.063-0.13	1	0.5	0.5
20	A82846C	1685.6	12.7	8-64	0.13-0.5	≤0.063	≤0.063	≤0.063
21	Orienticin A	1720.4	13.1	4-16	0.13-0.5	≤0.063	≤0.063	≤0.063

[a] Reported is the base peak (typically M+2 or M+3).[12]
[b] Analytical HPLC analysis of purified product, retention time reported in minutes.

Comparing the activity of the vancomycin derivatives (**14** and **18**) to the LY264826 derivatives (**4** and **19**) suggests the presence of the monosaccharide on amino acid residue 6 is important for activity against VRE, but does not improve the activity against staphylococci. The importance of the residue 6 chlorine substituent (indicated by *X* in Fig. 1) for activity against enterococci is evident by comparing compounds **15**, **16**, and **17** to compound **4**. Derivatives lacking a chlorine at this position (**15** and **16**) were found to be 4~8 times less active. On the other hand, loss of the residue 2 chlorine (indicated by *Y* in Fig. 1) had much less of an impact (compound **17**). It is also important to note that while the di-dechloro derivatives, **16** and **20**, are less active against enterococci as compared to the dichloro derivatives, **4** and **19**, they are 2~10 times more active against staphylococci. The divergent nature of the SAR may have implications on the mechanism of action of these semisynthetic glycopeptides and warrants further investigation.

Overall, semisynthetic modification of LY264826 provides the most potent activity against VRE and staphylococci. Against VRE, both **4** (LY307599) and **19** (LY309686) were as much as 50 times more active than their parent glycopeptide, and 4~16 times more active than the corresponding derivative of the other glycopeptides.

The present studies indicate that mono-alkylation of LY264826 on the disaccharide amino function affords semisynthetic glycopeptide derivatives with superior antimicrobial activity as compared to other alkylated products of the A82846 family. Two of the derivatives described, LY307599 and LY333328, are sufficiently active against enterococci and staphylococci to warrant further consideration as potential drug candidates. Complete details of an extensive SAR centered around the reductive alkylation of LY264826 will be the subject of future reports from this group.

Materials and Methods/Experimental

Antimicrobial Activity (*In Vitro*)

In vitro evaluation of staphylococci was carried out according to NCCLS recommendations for broth microdilution assay. Data for a single screening reference strain are shown. Strains shown are *Staphylococcus aureus* 489 (methicillin-resistant), *Staphylococcus epidermidis* 270, and *Staphylococcus haemolyticus* ST105.

In vitro evaluation of activity against enterococci was carried out in a screening assay using broth microdilution in Brain Heart Infusion medium. Strains used were four representitive vancomycin-resistant enterococci (two *E. faecium*, and two *E. faecalis*) with confirmed *vanA* genotypes, and five vancomycin-susceptible isolates (two *E. faecium*, and three *E. faecalis*). Comparative data are presented as the range of MIC's (μg/ml).

Experimental Procedures

General Procedure: The glycopeptide (1 mmol) was dissolved in 150 ml of methanol and treated with the aldehyde (1.1 mmol). The resulting solution was heated to 70°C for 2 hours then treated with $NaBH_3CN$ (1.1 mmol). After an additional 2 hours at 70°C, the

reaction mixture was cooled and the solvent removed *in vacuo*. The residue was purified by preparative HPLC. Alternatively, a solvent consisting of 50% methanol in DMF or water is acceptable.

Example 1 : Preparation of N-4-chlorobenzyl A82846B (**1**). The triacetate salt of A82846B (0.28 g, 0.157 mmol) was dissolved in 20 ml of DMF - CH_3OH, 1 : 1 (v/v), and treated with 4-chlorobenzaldehyde (40.5 mg, 0.288 mmol). The resulting mixture was heated to 70°C for 2 hours, then $NaBH_3CN$ (40.8 mg, 0.648 mmol) was added. Heating was continued at 70°C for an additional two hours, after which the mixture was cooled and concentrated *in vacuo*. Purification of the crude product by preparative HPLC afforded 0.109 g (40% yield) of **1**.

Example 2: Preparation of N-4-phenylbenzyl A82846B (**4**). A82846B free base (2.25 g, 1.41 mmol) was dissolved in 140 ml of DMF - CH_3OH, 1 : 1 (v/v), and treated with 4-biphenylcarboxaldehyde (0.331 g, 1.82 mmol). The resulting mixture was heated to 70°C for 2 hours, then $NaBH_3CN$ (0.554 g, 8.82 mmol) was added. Heating was continued at 70°C for an additional two hours, after which the mixture was cooled and concentrated *in vacuo*. Purification of the crude product by preparative HPLC afforded 1.02 g (41% yield) of **4**.

Example 3: Preparation of N-4'-chloro-4-phenylbenzyl A82846B (**9**). A82846B free base (1.0 g, 0.628 mmol) was dissolved in 100 ml of CH_3OH and treated with 4'-chlorobiphenyl-4-carboxaldehyde (0.131 g, 0.605 mmol). The resulting mixture was heated to 70°C for 2 hours, then $NaBH_3CN$ (46 mg, 0.73 mmol) was added. Heating was continued at 70°C for an additional two hours, after which the mixture was cooled and concentrated *in vacuo*. Purification of the crude product by preparative HPLC afforded 0.201 g (18% yield) of **9**.

Example 4: Modified procedure for the preparation of **9**, **10**, **11**, **12**, and **13**. A82846B free base (0.75 g, 0.47 mmol) was dissolved in 50 ml of CH_3OH - H_2O, 1 : 1 (v/v), and treated with 4'-chlorobiphenyl-4-carboxaldehyde (0.197 g, 0.91 mmol) and $NaBH_3CN$ (86 mg, 1.4 mmol). The resulting mixture was heated to reflux for 6.5 hours, after which the mixture was cooled and concentrated *in vacuo*. HPLC analysis (area percents, Zorbax SB-C_{18} column) of the crude reaction mixture afforded 14.9% **9**, 21.2% **10**, 25.9% **11**, 10.5% **12**, and 5.0% **13**.

HPLC Methods

Analytical: Reactions were monitored by analytical HPLC using a Waters μBondapak C_{18} column (3.9 × 300 mm) and UV detection at 280 nm. Elution was accomplished with a linear gradient of 5% CH_3CN - 95% buffer to 80% CH_3CN - 20% buffer over 30 minutes. The buffer used was 0.5% triethylamine in water, adjusted to pH 3 with H_3PO_4.

Preparative : Crude reaction mixtures were purified by preparative HPLC using a Waters C_{18} Nova-Pak column (40 × 300 mm) and UV detection at 280 nm. Elution was accomplished with a linear gradient of 5% CH_3CN - 95% buffer to 80% CH_3CN - 20% buffer over 30 minutes. The buffer used was 0.5% triethylamine in water, adjusted to pH 3 with H_3PO_4. The desired fractions were subsequently desalted with a Waters C_{18} Sep-Pak (35 cc) followed by lyophilization. Alternatively, a buffer containing 0.1% TFA in H_2O can be used, in which case the TFA salt is obtained directly after lyophilization.

FAB-MS Analysis

FAB-MS spectra were determined using a ZAB-2SE mass spectrometer. Samples were dispersed in thioglycerol, with added TFA, and introduced into the mass spectrometer on a FAB target.

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