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Exploring the Interest of 1,2-Dithiolane Ring System in Peptide Chemistry. Synthesis of a Chemotactic Tripeptide and X-ray Crystal Structure of a 4-Amino-1,2-dithiolane-4-carboxylic Acid Derivative

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Abstract—Due to their relevant biological functions and specific chemical reactivity 1,2-dithiolanes (five-membered cyclic disulfides) represent an emerging class of heterocyclic compounds. However, despite the extensive research centered on lipoic acid and its analogues, only very few data are at the present available on peptides containing this ring system. We report here synthesis, conformation and bioactivity of a fMLF-OMe analogue, namely For-Met-Adt-Phe-OMe (**7**), in which the residue of the 4-amino-1,2-dithiolane-4-carboxylic acid (Adt) (**4**) replaces the central L-leucine. The crystal conformation of the synthetic intermediate Boc-Adt-OMe (**5**) is also described and compared to that of lipoic acid (*R*-1,2-dithiolane-3-pentanoic acid) (**3**) and asparagusic acid (1,2-dithiolane-4-carboxylic acid) (**2**).

Introduction

The relevant role played by thiols and disulfides in biological systems is well known. The thiol–disulfide interchange reaction and the related formation and cleavage, under physiological conditions, of strong S–S bonds represent in fact one of the most versatile and efficient mechanisms through which the tertiary structure of polypeptides and proteins is controlled. However, the large majority of these structures involves linear or large ring disulfides; small ring systems, in which the S–S bond makes part of a conformationally constrained structure, are less frequently encountered. Two relevant, albeit different examples, which can be found among natural products, are represented by the eight-membered ring of the oxidized form of the -Cys-Cys- dyad **1** and the five-membered 1,2-dithiolane system of the lipoic acid (**3**). The cyclic form of the dipeptide **1** represents a well documented critical sequence in some proteins where it acts, with its opened and closed forms, as a molecular switch able to control receptor or enzyme activation.[1–4] The lipoic acid, on the other hand, is an extensively studied natural product which plays a dual role in living organisms: it is responsible for the transfer of acetyl groups from pyruvic acid to coenzyme A in the metabolic process which converts carbohydrates into energy and is a powerful antioxidant capable to defend the body against the free radical damage.[5–10]

During our studies on small ring disulfides and conformationally constrained analogues of cysteine we reported recently synthesis and properties of glutathione analogues incorporating the oxidized form of the -Cys-Cys- dyad **1** or the 4-amino-1,2-dithiolane-4-carboxylic acid (Adt) residue (**4**) in place of the native L-cysteine.[11,12] The interest for an α-aminoacid containing the 1,2-dithiolane ring system stems from the unique chemical properties shown by this structural fragment on which the biological functions of lipoic acid are based. Of particular relevance appear the fast rate of

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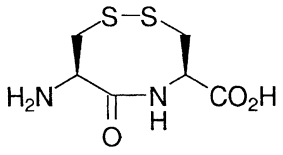
ring opening by nucleophiles as compared with related open-chain and cyclic disulfides,[13] the *trans*-acylating properties,[14] and the efficiency in the metal-ion coordination.[15,16] Although all these properties, as well as many others, have been the object of detailed studies, the incorporation of the 1,2-dithiolane ring system into the peptide backbone and the study of the chemical, conformational and biochemical consequences of this modification are just emerging in recent literature. Specifically, the so far available data concern in fact the glutathione analogue tripeptide γ-Glu-Adt-Gly-OH (**9**) in which the dithiolanic residue has been incorporated into the backbone through an isopeptidic bond (Scheme 1).[12]

As a development of our interest in this subject, we report here the crystal and molecular structure of the Boc-Adt-OMe (**5**) and the synthesis of **7**, an analogue of the prototypical chemotactic tripeptide *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine methyl ester (For-Met-Leu-Phe-OMe; fMLF-OMe) in which the central native Leu has been replaced by the Adt residue. The importance of the central aminoacidic residue in determining the activity of chemotactic fMLF-OMe analogues is well known and several position-2 modified analogues, incorporating $C^{\alpha,\alpha}$-dialkylglycines[17–20] as the Leu replacement, have been studied. In particular, it has been observed that the tripeptide For-Met-Ac_5c-Phe-OMe, containing the achiral 1-amino-1-cyclopentanecarboxylic acid (Ac_5c or cycloleucine) which is the cycloaliphatic analogue of the Adt, maintains the bioactivity of the parent while adopting a well defined β-turn backbone conformation.[21] On the basis of these findings, the synthesis of the new analogue For-Met-Adt-Phe-OMe (**7**) has been considered an interesting opportunity in order to gain information on two main points: (i) definition of the synthetic strategies suitable to incorporate the Adt residue into a peptide backbone and (ii) examination of the tendency of the five-membered cyclic disulfide residue to maintain the biological activity and the typical backbone folding which are encountered when the cyclopentane ring system is present at the central position of the bioactive tripeptide. Concerning the crystallographic analysis of Boc-Adt-OMe (**5**), an examination of the literature on 1,2-dithiolanes puts in clear evidence the importance of the steric arrangement of the bonds around the S–S group and the deriving sulfur lone-pair–lone-pair interaction for a complete understanding of the unusual reactivity of this class of small ring disulfides.[22–25] In this context, the here reported molecular structure can provide the first geometrical and conformational details of an orthogonally protected α-aminoacid residue whose C^{α} carbon atom makes part of the 1,2-dithiolane ring system; these structural parameters are compared with those concerning asparagusic acid (**2**),[26] lipoic acid (**3**),[27] and related literature available 1,2-dithiolanes.



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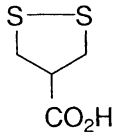

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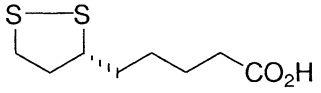

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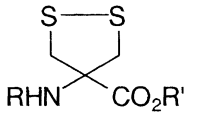
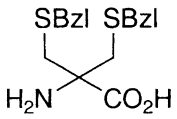


4 R = H; R' = H
5 R = Boc; R' = Me



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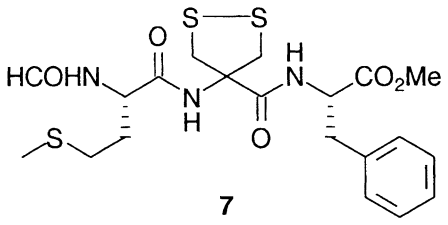

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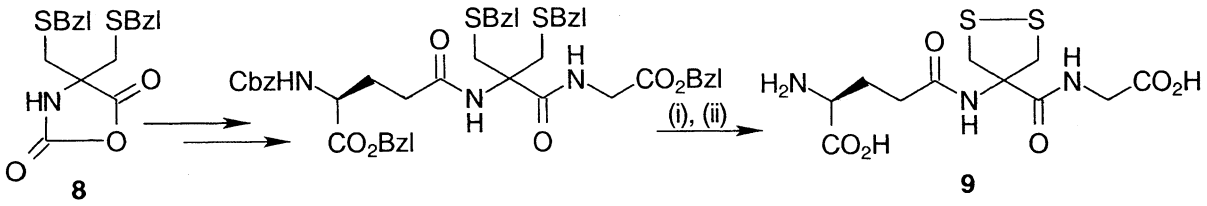
Results and Discussion

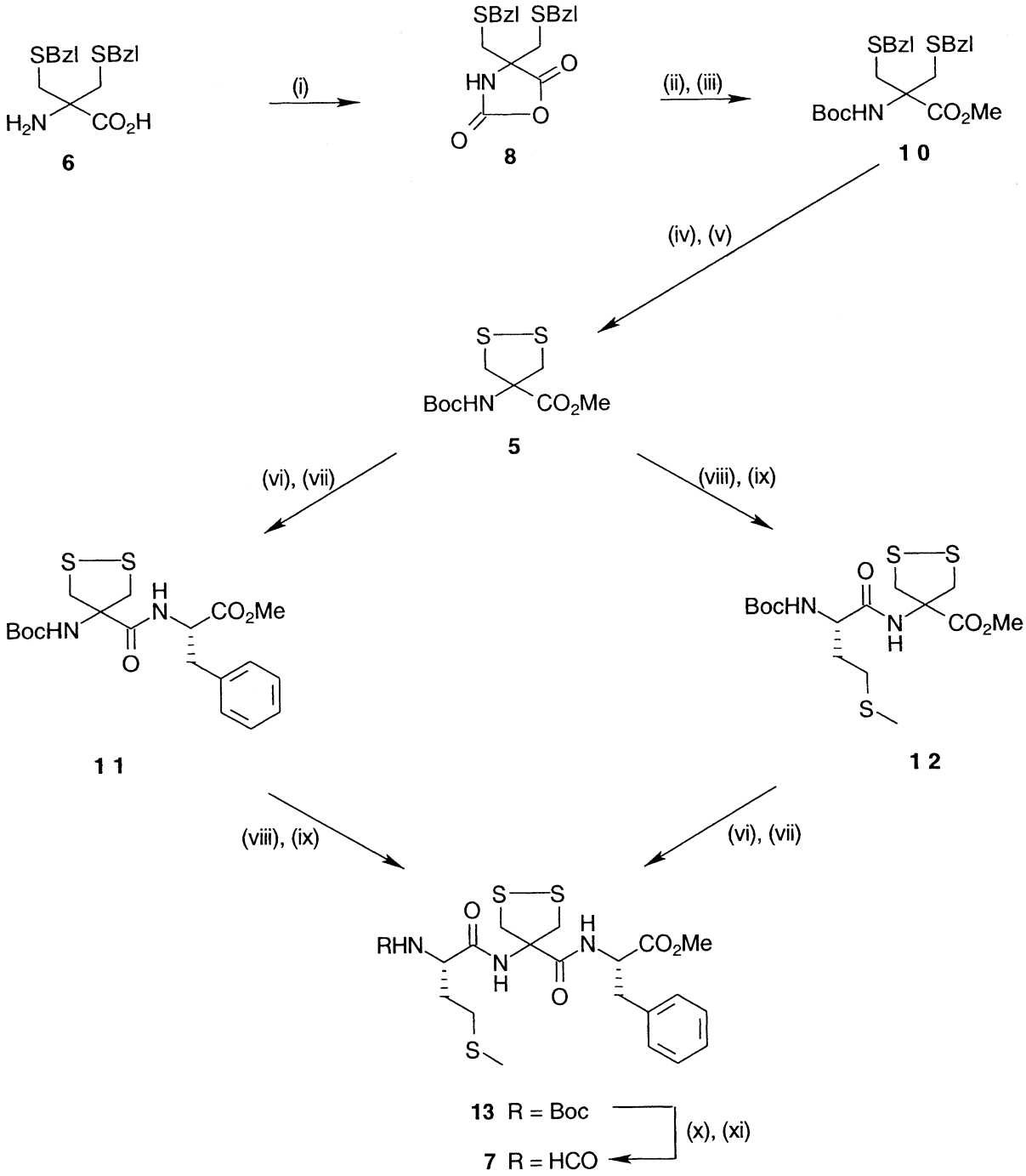
Chemistry

The products described in this paper have been synthesized according to Scheme 2; the here adopted strategy is different from that referring to the synthesis of the previously reported Adt containing glutathione analogue where the dithiolanic ring was formed only in the last step of the reaction sequence by deprotecting and oxidizing the residue of 2,2-bis-(benzylthiomethyl)-glycine (**6**) initially incorporated with the intermediacy of the corresponding *N*-carboxyanhydride **8** (Scheme 1).[12] In the case of the synthesis of the tripeptide under study For-Met-Adt-Leu-OMe (**7**), the above reported strategy cannot be followed due to the sensitivity of the methionine side-chain thioether function to the Na/liquid-NH_3 conditions requested to debenzylate the sulphydrylic groups of **6**.[28] Thus, as illustrated in Scheme 2, the methionine containing tripeptide **7** was obtained by following two new routes, both employing the orthogonally protected Boc-Adt-OMe derivative (**5**) as the starting product. However, the *N*-carboxyanhydride **8** plays a key role also in these two procedures, being the starting material from which **5** is obtained through *N*-carbamoylation followed by methanolysis.

By following the first route (see Scheme 2), the dithiolanic derivative Boc-Adt-OMe (**5**) is *C*-deprotected by mild aqueous alkaline hydrolysis and the resulting acid



Scheme 1. Reagents and conditions: (i) Na-liq NH_3, −78 °C; (ii) I_2, EtOH, 90% (two steps).



Scheme 2. Reagents and conditions: (i) $COCl_2$–toluene, THF, 88%; (ii) $(Boc)_2O$, Py, THF; (iii) MeOH, NMM, 93% (overall from **8**); (iv) Na-liq NH_3, −78 °C; (v) I_2, MeOH, 89% (overall from **10**); (vi) NaOH 2 N, MeOH; (vii) HOBt, EDC, H-Phe-OMe HCl, TEA, DMF, 84% (from **5**) and 82% (from **12**); (viii) $SOCl_2$, MeOH; (ix) HOBt, EDC, *N*-Boc-Met-OH, TEA, DMF, 60% (from **5**) and 59% (from **11**); (x) HCO_2H; (xi) EEDQ, DMF, 79% (from **13**).

is used for the coupling reaction with phenylalanine methyl ester to give the dipeptide **11**. In the alternative sequence the Boc-Adt-OMe derivative is first deprotected at the amino group by treatment with thionyl chloride in dry methanol and the resulting H_2N-Adt-OMe is *N*-acylated with Boc-Met-OH, by using EDC as the coupling reagent, to give the dipeptide methyl ester **12**. This was neatly hydrolyzed with methanolic NaOH and then coupled to H-Phe-OMe, by using the same condensing agent of the previous step, to afford the *N*-Boc-tripeptide **13**. This latter derivative could also be obtained by using, as the starting material, the dipeptide **11** and adopting analogous deprotecting and coupling procedures. In the last common step, the *N*-Boc tripeptide **13** is directly transformed into the corresponding *N*-Formyl analogue **7** by following the procedure of Lajoie and Kraus.[29] Despite its reactivity and in accordance with literature data on the reduced tendency of 4,4-disubstituted 1,2-dithiolanes to polymerize,[30] the dithiolanic residue is found stable under the adopted deprotection and coupling conditions and the yields, although not optimized, are in both cases satisfactory and nearly identical.

Solution conformation

To gain information on the conformational preferences of the Adt containing tripeptides **7** and **13**, the chemical shift dependence of the NH resonances as a function of DMSO-d_6 concentration in $CDCl_3$ solution (10 mM) was studied. The results are reported in Figure 1 where the resonances of the three NH groups of the tripeptides are shown.

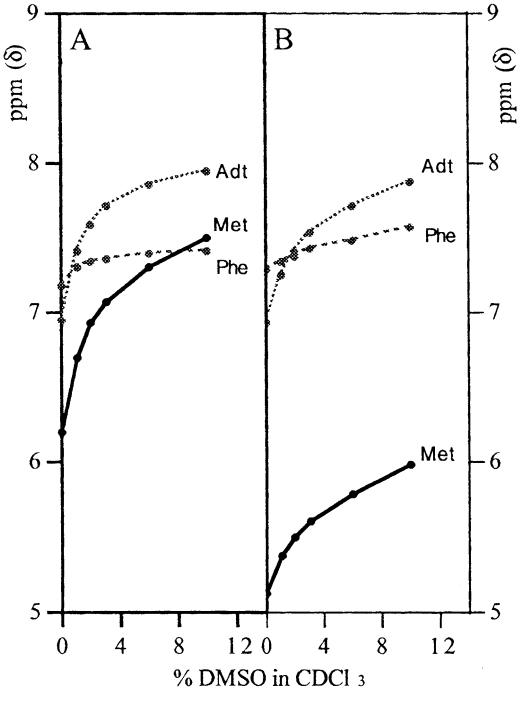


Figure 1. Plots of the NH chemical shifts (^{1}H NMR) as a function of the increasing DMSO-d_6 concentration (% v/v) in $CDCl_3$ solution (10 mM) for (A) the tripeptide For-Met-Adt-Phe-OMe (**7**) and (B) Boc-Met-Adt-Phe-OMe (**13**).

It is clearly seen that the Met and the Adt NH groups are significantly more sensitive to changes in solvent composition as compared with the Phe NH group which appears appreciably shielded. This result suggests that a significant population of conformers of the two tripeptides adopts, in $CDCl_3$ solution, a folded conformation characterized by an H-bond interaction between the Phe NH and a carbonylic group. An analogous situation has been evidenced in a series of conformationally restricted formyl-methionyl tripeptides of general formula HCO-Met-Xxx-Phe-OMe incorporating at the central position $C^{\alpha,\alpha}$-symmetrically disubstituted glycyl residues.[21] All these models show, together with their *N*-Boc precursors, a marked tendency to fold into β-turn conformations involving H-bond interaction between the Phe NH and the carbonyl group bound at the Met NH. In particular the DMSO-d_6 titration curve obtained by us with For-Met-Adt-Leu-OMe (**7**) (see Fig. 1A) shows practically the same values exhibited by the *N*-formyltripeptide For-Met-Ac_5c-Leu-OMe.[21]

Further information on the solution conformations and the type of β-turn involved can be obtained by examining the results of nuclear Overhauser effect (NOE) experiments summarized in Table 1. It should be recalled that in standard β-turns the interproton distances which differentiate type-I from type-II structures are $C_{i+1}H$–$N_{i+2}H$ (3.4 and 2.2 Å in type I and II, respectively) and $N_{i+1}H$–$N_{i+2}H$ (2.6 and 4.5 Å in type I and II, respectively); furthermore, a short distance of 2.4 Å between $N_{i+2}H$–$N_{i+3}H$ is observed in both the types of turns.[31] As shown in Table 1 the two peptides **7** and **13** show strong sequential NOEs Met $C^{\alpha}H$ Adt NH; this, together with the Phe NH Adt NH NOEs and the absence of connections between Adt NH and Met NH, clearly supports the presence of type-II β-turns with the Adt residue located at $i+2$ position. In this type of β-turns, contrarily to type I, the central N–H amide bond (residue $i+2$) is *cis* to the $C^{\alpha}H$ of the preceding residue ($i+1$) and this conformational feature is reflected by the strong NOE observed between the Met $C^{\alpha}H$ and the Adt NH of both **7** and **13**. Fig. 2 illustrates the proposed folding by putting also in evidence the intramolecular hydrogen bond ($i+3 \rightarrow i$) between H–CO (or Bu′OCO) and Phe NH closing a pseudocyclic 10-membered ring.

Table 1. Observed nuclear Overhauser effects (NOEs) in the NOESY spectra of HCO-Met-Adt-Phe-OMe (**7**) and Boc-Met-Adt-Phe-OMe (**13**)[a]

	7	**13**
Met NH...H–CO	s	—
Met NH...Adt NH	—	—
Met $C^{\alpha}H$...Met $C^{\gamma}H_2$	s	m
Met $C^{\alpha}H$...Met $C^{\beta}H_2$	s	m
Met $C^{\alpha}H$...Adt NH	s	s
Adt NH...Adt CH_2	m	m
Phe NH...Adt NH	m	w
Phe NH...Adt CH_2	m	m
Phe NH...Phe $C^{\beta}H_2$	w	w
Phe NH...Phe $C^{\alpha}H$	s	s
Phe $C^{\alpha}H$...Phe $C^{\beta}H_2$	s	s
Phe $C^{\beta}H_2$...aromatic–H	s	s

[a]s, strong; m, medium; w, weak.

A final consideration concerns the NOE between Phe NH and Adt NH which, in the case of the *N*-Boc tripeptide **13**, is found to be weaker than that observed in the *N*-For tripeptide **7** (see Table 1). This finding could indicate that a H-bond interaction, involving the Met CO instead of the Bu^{t}OCO as acceptor could be operative in **13**. An examination of the models shows in fact that this latter H-bond interaction, closing a seven-membered pseudocyclic ring (γ-turn), is associated with a larger interproton Phe NH–Adt NH distance as compared with the β-turn.[32]

Conformation in the crystal of Boc-Adt-OMe (5)

Figure 3 reports a perspective view of the conformation in the crystal of Boc-Adt-OMe (**5**) together with the numbering scheme usually adopted for aminoacid derivatives and peptides. As can be deduced by the figure and by the data in Table 2, the Boc–NH–chain adopts a *trans–trans* planar conformation and shows a C_{04}–C_{01}–O_{01} valence angle (102.3°) smaller by about 7° than the tetrahedral value; both these findings are in accordance with the geometrical and conformational peculiarities derived from crystallographic investigations on peptides containing the *tert*-butyloxycarbonylamino group.[33] Considering the geometrical dimension of the five-membered ring it can be seen that the two C–S bond lengths are nearly identical and the same is true for the two C–C bonds. A quite different situation concerns the intraring valence angles at the sulfur atoms which show significantly different values, being 91.4 and 97.2°, at $S_1{}^{\gamma 1}$ and $S_1{}^{\gamma 2}$, respectively. The S–S bond length (2.056 Å) corresponds to that found in the case of lipoic acid (2.053 Å) and is longer than that found in strain-free open-chain disulfides (ca. 2.044 Å).

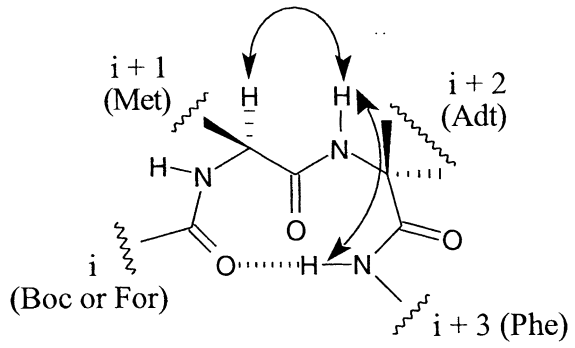


Figure 2. Relevant interproton correlations as deduced by NOESY experiments for peptides **7** and **13**.

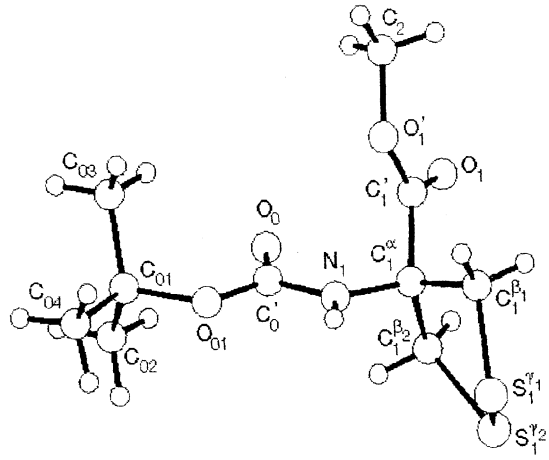


Figure 3. A perspective view of the crystal conformation of Boc-Adt-OMe (**5**).

Further relevant features in the structure of **5** are the non planarity of the ring and the torsion angle around the S–S bond. An examination of Figure 3 reveals that the dithiolane ring of **5** can be described as an envelope with four atoms, including the sulfur bridge, on a plane and one carbon atom (the $C_1{}^{\beta 1}$) at the envelope flap; this latter atom is oriented on the opposite side of the Boc–NH substituent and bound at the sulfur atom $S_1{}^{\gamma 1}$ which exhibits the smaller intraring valence angle. The degree of deviation from planarity of the ring can be evaluated from the distance between the $C_1{}^{\beta 1}$ and the mean plane of the other four ring atoms which was found to be 0.76 Å. On the basis of these features a pseudomirror plane, bisecting the $C_1{}^{\beta 2}$-$S_1{}^{\gamma 2}$ bond and passing through the carbon at the flap, can be individuated. A final observation concerns the valence angle N_1–$C_1{}^{\alpha}$–$C_1{}'$ (τ angle; see Table 2) whose value, in peptides containing $C^{\alpha,\alpha}$-symmetrically substituted glycines, is highly sensible to the type of adopted local conformation and is always smaller than the tetrahedral value in the fully-extended conformations (typical values: 102–107°).[34,35] The value here found for the Adt derivative **5** is 110.1° and is in perfect accordance with the value observed in Ac_5c containing peptides and related folded structures.[34]

In order to compare the above reported data with those of already known 1,2-dithiolanes, an examination of the literature has been performed by taking into consideration saturated rings not fused with other cyclic systems and containing disulfide groups not involved in metal ion complexes. Whereas several X-ray structures of 3-monosubstituted-1,2-dithiolanes related to lipoic acid are available, only asparagusic acid structure has been found as representative of 4-monosubstituted derivatives.[26] Five 1,2-dithiolane structures concern 3,4-

Table 2. Relevant geometrical and conformational data of Boc-Adt-OMe (**5**)[a]

Bond lengths (Å)		Torsion angles (°)[b]	
$S_1{}^{\gamma 1}$-$S_1{}^{\gamma 2}$	2.056(3)	Main chain	
$S_1{}^{\gamma 1}$-$C_1{}^{\beta 1}$	1.806(6)		
$S_1{}^{\gamma 2}$-$C_1{}^{\beta 2}$	1.809(6)	O_{01}-C'_0-N_1-$C_1{}^{\alpha}$	177.1(5)
$C_1{}'$-$C_1{}^{\alpha}$	1.534(8)	C'_0-N_1-$C_1{}^{\alpha}$-$C_1{}'$	50.1(6)
$C_1{}^{\alpha}$-$C_1{}^{\beta 2}$	1.555(8)	N_1-$C_1{}^{\alpha}$-$C_1{}'$-$O_1{}'$	45.3(6)
$C_1{}^{\alpha}$-$C_1{}^{\beta 1}$	1.539(8)	$C_1{}^{\alpha}$-$C_1{}'$-$O_1{}'$-C_2	179.7(5)
Valence angles (°)		1,2 Dithiolane ring	
$C_1{}^{\beta 1}$-$S_1{}^{\gamma 1}$-$S_1{}^{\gamma 2}$	91.4(2)	$C_1{}^{\alpha}$-$C_1{}^{\beta 1}$-$S_1{}^{\gamma 1}$-$S_1{}^{\gamma 2}$	47.8(4)
$C_1{}^{\beta 2}$-$S_1{}^{\gamma 2}$-$S_1{}^{\gamma 1}$	97.2(2)	$C_1{}^{\alpha}$-$C_1{}^{\beta 2}$-$S_1{}^{\gamma 2}$-$S_1{}^{\gamma 1}$	10.5(4)
$C_1{}^{\beta 1}$-$C_1{}^{\alpha}$-$C_1{}^{\beta 2}$	109.3(5)	$C_1{}^{\beta 1}$-$S_1{}^{\gamma 1}$-$S_1{}^{\gamma 2}$-$C_1{}^{\beta 2}$	−31.5(3)
N_1-$C_1{}^{\alpha}$-$C_1{}'$	110.1(4)	$C_1{}^{\beta 1}$-$C_1{}^{\alpha}$-$C_1{}^{\beta 2}$-$S_1{}^{\gamma 2}$	20.6(6)
$S_1{}^{\gamma 2}$-$C_1{}^{\beta 2}$-$C_1{}^{\alpha}$	110.6(5)	$C_1{}^{\beta 2}$-$C_1{}^{\alpha}$-$C_1{}^{\beta 1}$-$S_1{}^{\gamma 1}$	−47.8(5)
$S_1{}^{\gamma 1}$-$C_1{}^{\beta 1}$-$C_1{}^{\alpha}$	107.3(4)		
C_{04}-C_{01}-O_{01}	102.3(5)		

[a]Atom numbering is that reported in Figure 3.
[b]Torsion angles have been computed according to ref 44.

disubstituted derivatives and have been determined in connection with the assignment of the stereochemistry to gerrardine[36] and guinesine[37,38] alkaloids. No structures referring to 4,4-disubstituted rings have been found. The analysis of the data shows that the S–S bond length and the torsion angle of 3-monosubstituted derivatives fall in the range of 2.03–2.06 Å and −29.5 to −35.3°, respectively.

Examination of these values, together with those found for asparagusic acid and reported in Table 3, shows that S–S bond length and torsion angle of the Boc-Adt-OMe (**5**) (2.056 Å and 31.5°) are more consistent with 3-monosubstituted derivative structures, including lipoic acid, than with the 4-monosubstituted model represented by the asparagusic acid. This latter compound presents in fact a lower S–S torsion angle (26.6°) and a longer S–S bond length (2.10 Å). A different picture is presented by the 3,4-disubstituted models as esemplified by the two diastereoisomeric forms of a guinesine derivative containing the 3,4-disubstituted-1,2-dithiolane ring. Here the *cis* 3,4-isomer exhibits usual values of S–S bond length and torsion angle (2.04 Å and 36.4°) whereas the corresponding values of the *trans* 3,4-isomer are 2.09 Å and −2.6°, respectively. Thus, this compound shows the smallest value of S–S torsion angle observed in X-ray crystal structures of 1,2-dithiolanes and underlines, at the same time, the strong influence of the substitution pattern on the ring conformation.

Structure–reactivity relationship

An interesting correlation between the decreasing of the torsion angle around the disulfide bridge with the increasing of the S–S bond length has been evidenced by Bock et al.[22] It is now well established that the major contribution to the enhanced reactivity of 1,2-dithiolanes is given by the small dihedral angle around the S–S bond associated with the reduced size of the ring and by the consequent unfavourable interaction between the adjacent sulfur lone-pairs.[25] Accordingly, the S–S bond in 1,2-dithiolanes is longer and weaker than that found in open chain disulfides whose dihedral angle is ca. 90° and the S–S bond length about 2.02 Å. The interest and the implication of this finding should be evaluated in the light of the literature data which register the different bioactivity shown by the oxidized forms of glutathione GSSG (a constraint-free linear disulfide) and oxidized lipoic acid (a five-membered cyclic disulfide).[8,39] The appropriate example in this context is the ability to scavenge hypochlorous acid, a dangerous oxidant of several biological systems: whereas the reduced forms of both GSH and lipoic acid are potent HClO scavengers, only the oxidized (1,2-dithiolane form) of lipoic acid is able to quench the oxidant activity of HClO, the GSSG being practically inactive. This quite surprising finding can be explained by taking into account the different nature of the S–S bond in the two molecules.[8,39]

Table 3. Structural parameters characterizing lipoic acid (**3**), asparagusic acid (**2**), and *N*-Boc-Adt-OMe (**5**)[a]

	Lipoic acid[b]	Asparagusic acid[c]	Boc-Adt-OMe
Bond length (Å)			
C–S	1.83	1.83	1.806
C–S*	1.79	1.85	1.809
S–S	2.053	2.10	2.056
Intraring valence angle (°)			
S	92.8	92.6	91.4
S*	95.5	96.6	97.2
Torsion angle (°)			
C–S–S*–C	35.0	26.6	31.5

[a]The asterisk indicates the sulfur atom with the major intraring valence angle.
[b]See ref 27.
[c]See ref 26.

Biological activity

The biological activity of the Adt containing tripeptide **7** was determined on human neutrophils and compared with that of the prototypical bioactive agent fMLF-OMe. Directed migration (chemotaxis), lysozyme release, and superoxide anion production have been measured. As shown in Figure 4A, compound **7** is active in inducing neutrophil chemotaxis, even though with lower potency than the reference model. The activity as secretagogue agent on the lysozyme release (Fig. 4B) and in terms of superoxide anion production (Fig. 4C) remains very low at concentrations ranging from 10^{-9} to 10^{-7} M but increases rapidly, reaching the value shown by the reference tripeptide, starting from 10^{-5} M.

Conclusions

In the present paper, we reported synthetic strategies which allow the direct incorporation of a 1,2-dithiolanic aminoacid residue into peptide backbones. The results show that the achiral and conformationally constrained Adt, possessing a quaternary carbon atom at position 4 of the dithiolanic ring, is stable under the conventional reaction conditions adopted during peptide synthesis. Furthermore, the incorporation of such residue at the central position of a bioactive tripeptide, leads to an analogue which maintains the bioactivity and induces a backbone folding analogous to that imposed by the Ac_5c, the $C^{\alpha\alpha}$-tetrasubstituted aminoacid which is the dicarba analogue of the Adt. This latter finding is in accordance with literature data concerning isosterism and bioisosterism of disulfides, a topic relevant to the development of cystine isosteres; it is well known in fact that the most frequently exploited cystine isostere is its dicarba analogue (2*S*,7*S*)-2,7-diaminosuberic acid.[40] Biological active peptides containing this residue have been developed in order to improve the stability and obtain analogues devoid of the chemical and metabolic lability typical of the disulfide bonds. The here reported results suggest an inverse isosteric relationship concerning the couple Adt/Ac_5c whose interest lies on the possibility to exploit the chemical versatility offered by the constrained disulfide system including the hydrogenolytic transformation into $C^{\alpha,\alpha}$-disubstituted cysteine analogues.[12]

Finally, a comparison of the structural parameters of the molecule of Boc-Adt-OMe (**5**) with those of asparagusic acid shows that the presence of two substituents on C-4 carbon atom of the 1,2-dithiolane ring, as compared with the monosubstitution on the same atom present in the asparagusic acid, favours the S–S bonding interaction, thus leading to a stereoelectronic arrangement which closely resembles that found in lipoic acid (**3**). This finding, together with the stability during the synthetical steps and the properties shown by the tripeptide **7**, stimulates further studies on peptides containing 1,2-dithiolane ring systems and in particular the 4,4-disubstituted and achiral Adt residue; the availability and the possible exploitation of peptide models possessing lipoic acid related properties, seem in fact a rewarding objective.

Studies on further aminoacids and peptides, characterized by the presence of the 1,2-dithiolane ring, are at present in progress in our laboratories.

Experimental

Melting points were determined with a Kofler hot stage apparatus and are uncorrected. Optical rotations were taken at 20 °C with a Schmidt-Haensch Polartronic D polarimeter in a 1 dm cell ($CHCl_3$). Infrared spectra were recorded with a Perkin-Elmer 983 spectrophotometer. ^1H and ^{13}C NMR spectra were measured with a Varian Mercury 300 spectrometer using tetramethylsilane as internal standard. NOESY spectra were recorded in $CDCl_3$ containing 5.0% of DMSO-d_6 on an Avance 2000 Bruker console with a 1200-ms mixing time and displayed in the phase-sensitive mode. Usually 256×1024 data points were collected and for each block 64 transients were collected for two-dimensional experiments. The data sets were linearly predicted to 512×1024 data points. A Gaussian window was applied in both dimensions. Zero filling was used for a final spectrum size of 1024×1024 data points. Analytical results were within ±0.4% of the theoretical values. Column chromatographies were carried out using Merck silica gel 60 (230–400 mesh). Thin layer chromatographies were performed on silica gel Merck 60 F_{254} plates. The drying agent was sodium sulfate. Following abbreviations are used for reagents and solvents: Boc_2O (di-*t*-butyldicarbonate), DMF (*N,N*-dimethylformamide), EDC (*N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride), EEDQ (*N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline), HOBt (1-hydroxybenzotriazole), NMM (4-methylmorpholine), TEA (triethylamine). All reagents and solvents were purchased at highest commercial quality and used without further purification, except for dry ethyl ether and tetrahydrofuran (THF) which were distilled from $LiAlH_4$.

***N*-Carboxy-2,2-bis(benzylthiomethyl)glycine anhydride (NCA) (8).** To a stirred suspension of 2,2-bis(benzylthiomethyl)glycine (**6**)[41] (0.500 g, 1.44 mmol) in dry THF (20 mL) 4.5 mL of a 1.93 M phosgene solution in toluene (Fluka 79380) were added dropwise at 0 °C. After 2 h at room temperature, the solvent was removed under reduced pressure below 40 °C and the resulting residue (0.505 g), dissolved in dry ethyl ether, was filtered on a short pad of silica gel to give pure NCA **8** (0.472 g, 88%).[12]

***N*-Boc-2,2-bis(benzylthiomethyl)-Gly-OMe (10).** The NCA **8** (0.485 g, 1.3 mmol) was dissolved in dry THF (3 mL) and the solution was then cooled to −15 °C with stirring. Boc_2O (0.340 g, 1.56 mmol) in dry THF (2 mL)

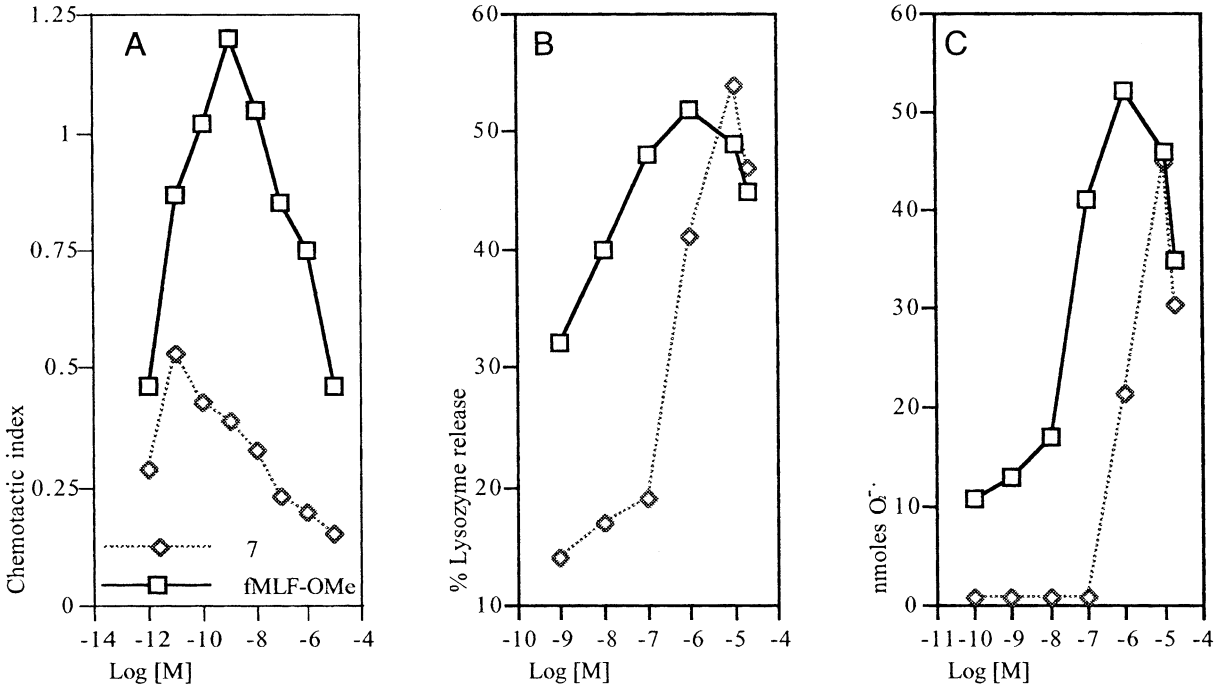


Figure 4. Biological activity for For-Met-Adt-Phe-OMe (**7**) towards human neutrophils: (A) chemotactic activity; (B) release of neutrophil granule enzymes evaluated by determining lysozyme activity; (C) superoxide anion production.

and dry pyridine (0.21 mL, 2.6 mmol) were added successively and the resulting mixture was stirred 10 min at −15 °C and 20 h at room temperature, at which time the reaction was deemed complete by TLC (CH_2Cl_2). Dry MeOH (1.5 mL) and NMM (0.286 mL, 2.6 mmol) were added to the mixture and the stirring was continued at room temperature for further 24 h. After removing all volatile components under reduced pressure, the residual syrup (0.675 g) was chromatographed on silica gel (20 g) using CH_2Cl_2/hexane = 6/4 as eluent to give pure **10** as a waxy oil (0.560 g, 93%).[12]

***N*-Boc-Adt-OMe (5).** Compound **10** (1.491 g, 3.23 mmol) was dissolved at −78 °C in approximately 140 mL of freshly distilled liquid NH_3, carefully excluding moisture. Then Na was slowly added (ca. 0.35 g) in small portions until the blue colour of the solution persisted for 10 min. Ammonium chloride (0.814 g, 15.2 mmol) was added and the solvent allowed to evaporate under a stream of dry nitrogen. The residue was dilute with MeOH (100 mL), neutralized with 2 N HCl, and additioned dropwise with an aqueous solution of iodine 0.1 M until a persistent pale yellow colour appeared. The solution was decolourized with 1 M $Na_2S_2O_3$, concentrated under reduced pressure, diluted with water, and extracted with EtOAc. The organic phase was washed with water, dried, and evaporated to give crude **5** (0.889 g) which was further purified by crystallization from MeOH (0.801 g, 89%). Mp 104–105 °C.[12]

***N*-Boc-Adt-Phe-OMe (11).** To a solution of **5** (0.213 g, 0.76 mmol) in MeOH (3.5 mL) 2N NaOH (0.76 mL) was added under stirring. After 6 h at room temperature, the solution was concentrated under vacuum, acidified with 0.5 N HCl, and extracted with EtOAc; the organic phase was washed with water, dried, and evaporated. The resulting residue (0.206 g) was used in the next step without further purification.

To the previous residue, dissolved in DMF (2.0 mL) and cooled to 0 °C, HOBt (0.130 g, 0.84 mmol) and EDC (0.161 g, 0.84 mmol) were added. After stirring for 15 min at 0 °C and for 20 min at room temperature, H-Phe-OMe·HCl (0.181 g, 0.84 mmol) and TEA (0.118 mL, 0.84 mmol) were added. The reaction mixture was stirred at room temperature for 18 h and then diluted with water and extracted with EtOAc. The organic phase was washed with water, 10% citric acid solution, saturated $NaHCO_3$, and water, dried, and evaporated under reduced pressure. The residue (0.310 g) was chromatographed on silica gel (10 g) using $CHCl_3$/EtOAc = 95:5 as eluent to give 0.271 g of **11** (84%). Mp 193–195 °C (from acetone); $[\alpha]_D$ + 39° (*c* 1.0); IR ($CHCl_3$) 3411, 1720, 1682, 1505, 1369, 1158 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.43 (s, 9H, *t*-butyl), 3.09 (dd, 1H, *J* = 6.5 and 14.1 Hz, Phe β-CH_A), 3.18 (dd, 1H, *J* = 5.7 and 14.1 Hz, Phe β-CH_B), 3.43 (m, 4H, 2×Adt β-CH_2), 3.72 (s, 3H, OCH_3), 4.89 (m, 1H, Phe α-CH), 5.29 (s, 1H, Adt NH), 7.10–7.29 (m, 6H, aromatics and Phe NH); ^{13}C NMR (75.43 MHz, $CDCl_3$) δ 28.1 [(C(*C*H_3)$_3$], 37.9 (Phe β-CH_2), 46.4 (2×CH_2S), 52.4 (Phe α-CH), 53.5 (OCH_3), 71.7 (Adt α-C), 81.3 [(*C*(CH_3)$_3$], 127.2, 128.6, 129.2, 135.7 (aromatics), 154.5, 169.4, 171.6 (3×CO). Anal. $C_{19}H_{26}N_2O_5S_2$ (426.54), C, H, N, S.

***N*-Boc-Met-Adt-OMe (12).** To a solution of **5** (0.200 g, 0.72 mmol) in dry MeOH (5.0 mL) cooled at 0 °C thionyl chloride (0.052 mL, 0.72 mmol) was added and the mixture was stirred at 50 °C for 3 h. Evaporation under reduced pressure of the solution afforded H-Adt-OMe·HCl (0.160 g). To the stirred suspension of the hydrochloride in dry DMF (2 mL) cooled at 0 °C *N*-Boc-Met-OH (0.197 g, 0.79 mmol), HOBt (0.122 g, 0.79 mmol), EDC (0.151 g, 0.79 mmol), and, after 15 min at 0 °C and 20 min at room temperature, TEA (0.100 mL, 0.72 mmol) were added. The mixture was stirred at room temperature for 18 h and then worked up as described for **11** to give a residue (0.238 g) which, chromatographed on silica gel (10 g) using hexane/EtOAc = 65:35 as eluent, afforded 0.178 g of **12** (60%). Mp 132–134 °C (from CH_2Cl_2/hexane); $[\alpha]_D$ −21° (*c* 1.0); IR ($CHCl_3$) 3341, 1744, 1710, 1497, 1368, 1292, 1163 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.46 (s, 9H, *t*-butyl), 1.96 (m, 1H, Met β-CH_A), 2.07 (m, 1H, Met β-CH_B), 2.12 (s, 3H, S-CH_3), 2.60 (m, 2H, Met γ-CH_2), 3.40 (m, 2H, Adt 2×CH_A), 3.65 (apparent d, 2H, Adt 2×CH_B), 3.78 (s, 3H, OCH_3), 4.30 (m, 1H, Met α-CH), 5.20 (d, 1H, *J* = 7.5 Hz, Met NH), 7.17 (s, 1H, Adt NH); ^{13}C NMR (75.43 MHz, $CDCl_3$) δ 15.3 (Met CH_3), 28.3 [(C(*C*H_3)$_3$], 30.0 (Met γ-CH_2), 30.8 (Met β-CH_2), 47.2, 47.4 (2×Adt β-CH_2), 52.9 (Met α-CH), 53.3 (OCH_3), 70.8 (Adt α-C), 80.5 [(*C*(CH_3)$_3$], 155.7, 170.0, 171.4 (3×CO). Anal. $C_{15}H_{26}N_2O_5S_3$ (410.56), C, H, N, S.

***N*-Boc-Met-Adt-Phe-OMe (13).** From **11**: to a solution of **11** (0.187 g, 0.44 mmol) in dry MeOH (3.0 mL) cooled at 0 °C thionyl chloride (0.032 mL, 0.44 mmol) was added and the mixture was stirred at 50 °C for 5 h. Evaporation under reduced pressure of the solution afforded H-Adt-Phe-OMe·HCl as a foam. To the stirred suspension of the hydrochloride in dry DMF (1.5 mL) cooled at 0 °C *N*-Boc-Met-OH (0.120 g, 0.48 mmol), HOBt (0.074 g, 0.48 mmol), EDC (0.092 g, 0.48 mmol), and, after 15 min at 0 °C and 20 min at room temperature, TEA (0.062 mL, 0.44 mmol) were added. The mixture was stirred at room temperature for 20 h and then worked-up as above. Chromatography of the residue (0.221 g) on silica gel (7 g) using $CHCl_3$/EtOAc = 9:1 as eluent afforded 144 mg of **13** (59%). Mp 164–165 °C (from acetone); $[\alpha]_D$ −13° (*c* 1.0); IR ($CHCl_3$) 3340, 1740, 1692, 1492, 1437, 1367, 1162 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.43 (s, 9H, *t*-butyl), 1.94 (m, 1H, Met β-CH_A), 2.09 (m, 1H, Met β-CH_B), 2.11 (s, 3H, SCH_3), 2.59 (m, 2H, Met γ-CH_2), 3.06 (dd, 1H, *J* = 7.2 and 14.1 Hz, Phe β-CH_A), 3.19 (dd, 1H, *J* = 6.0 and 14.1 Hz, Phe β-CH_B), 3.49 (m, 4H, 2×Adt β-CH_2), 3.71 (s, 3H, OCH_3), 4.22 (m, 1H, Met α-CH), 4.83 (m, 1H, Phe α-CH), 5.22 (d, 1H, *J* = 7.2 Hz, Met NH), 6.98 (s, 1H, Adt NH), 7.12–7.31 (m, 6H, aromatics and Phe NH); ^{13}C NMR (75.43 MHz, $CDCl_3$) δ 15.3 (Met CH_3), 28.3 [(C(*C*H_3)$_3$], 30.2 (Met β and γ-CH_2), 37.7 (Phe β-CH_2), 45.9, 46.8 (2×Adt β-CH_2), 52.4 (Met and Phe α-CH), 53.9 (OCH_3), 71.7 (Adt α-C), 80.8 [(*C*(CH_3)$_3$],

127.1, 128.6, 129.2, 136.0 (aromatics), 155.9, 169.0, 171.6, 171.9 (4×CO). Anal. $C_{24}H_{35}N_3O_6S_3$ (557.74), C, H, N, S.

From **12**: to a solution of **12** (0.150 g, 0.37 mmol) in MeOH (2.0 mL), 2 N NaOH (0.37 mL) was added under stirring. After 6 h at room temperature, the solution was concentrated under vacuum below 30 °C, acidified with 0.5 N HCl, and extracted with EtOAc; the organic phase was washed with water, dried, and evaporated. The resulting residue (0.153 g) was used in the next step without further purification.

To the previous residue dissolved in DMF (2.0 mL) and cooled to 0 °C, HOBt (0.063 g, 0.41 mmol) and EDC (0.078 g, 0.41 mmol) were added. After stirring 15 min at 0 °C and 20 min at room temperature, H-Phe-OMe·HCl (0.088 g, 0.41 mmol) and TEA (0.057 mL, 0.41 mmol) were added. The reaction mixture was stirred at room temperature for 18 h and then worked up as described for **11** to give a residue (0.170 g) which, chromatographed on silica gel (7 g) using $CHCl_3$/EtOAc = 95:5 as eluent, afforded 0.154 g of **13** (82%) identical to the sample obtained from **11**.

HCO-Met-Adt-Phe-OMe (7). A solution of **13** (0.097 g, 0.174 mmol) in 0.7 mL of 98% formic acid was stirred at room temperature for 18 h. After removal of the excess of formic acid under vacuum, the residue was dissolved in dry DMF (0.7 mL) and EEDQ (0.052 g, 0.209 mmol) was added. The solution was stirred at room temperature for 18 h. Evaporation under reduced pressure afforded a residue (0.168 g) which was chromatographed on silica gel (5 g) using $CHCl_3$/EtOAc = 6:4 as eluent to give pure **7** (0.067 g, 79%). Mp 112–116 °C (from acetone); $[\alpha]_D$ −20° (*c* 1.0); IR ($CHCl_3$) 3319, 1742, 1673, 1494, 1436, 1360 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.98 (m, 1H, Met β-CH_A), 2.10 (m, 1H, Met β-CH_B), 2.12 (s, 3H, SCH_3), 2.62 (m, 2H, Met γ-CH_2), 3.08 (dd, 1H, *J* = 7.2 and 14.1 Hz, Phe β-CH_A), 3.19 (dd, 1H, *J* = 5.8 and 14.1 Hz, Phe β-CH_B), 3.38, 3.50 (ABq, 2H, *J* = 12.4 Hz, Adt C_AH_2), 3.60 (s, 2H, Adt C_BH_2), 3.72 (s, 3H, OCH_3), 4.61 (m, 1H, Met α-CH), 4.84 (m, 1H, Phe α-CH), 6.21 (d, 1H, *J* = 6.3 Hz, Met NH), 6.95 (s, 1H, Adt NH), 7.11–7.32 (m, 6H, aromatics and Phe NH), 8.10 (s, 1H, HCO); ^{13}C NMR (75.43 MHz, $CDCl_3$) δ 15.3 (Met CH_3), 30.0 (Met γ-CH_2), 30.9 (Met β-CH_2), 37.6 (Phe β-CH_2), 45.9, 47.3 (2×Adt β-CH_2), 51.4 (Met α-CH), 52.4 (Phe α-CH), 53.9 (OCH_3), 71.9 (Adt α-C), 127.1, 128.5, 129.3, 136.0 (aromatics), 161.8, 169.2, 171.2, 171.9 (4×CO). Anal. $C_{20}H_{27}N_3O_5S_3$ (485.63), C, H, N, S.

X-ray single crystal analysis

Crystals were obtained by slow evaporation of a methanol solution of **5**. X-ray data (see Table 4) were collected at room temperature on a Rigaku AFC5R diffractometer equipped with a graphite monochromated Cu-K_α radiation (λ = 1.5418 Å) and 12 KW rotating anode source.

Reflections with $I < 15\sigma(I)$ were rescanned up to four times with an accumulation of counts to improve counting statistics. An empirical absorption correction, based on azimuthal scans of several reflections, was applied. The data were finally corrected for Lorentz and polarization effects. The structure was solved by direct methods. The non-H atoms were refined anisotropically by full-matrix least-squares method. The H atoms were located at the expected positions and included in the last structure factors calculations with isotropic thermal parameters deduced from the carrier atoms. All the calculations were performed using TEXSAN crystallographic software package.[42]

Biological assay

Cells. Human periferal blood neutrophiles were purified employing the standard techniques of dextran (Pharmacia, Uppsala, Sweden) sedimentation, centrifugation on Ficoll-Paque (Pharmacia), and hypotonic lysis of red cells. The cells were washed twice and resuspended in KRPG (Krebs–Ringer phosphate containing 0.1% w/v glucose, pH 7.4) at a concentration of 50×10^6 cells/mL. Neutrophils were 98–100% pure.

Random locomotion. Random locomotion was performed with 48-well microchemotaxis chamber (Bio Probe, Milan, Italy) and the migration into the filter was evaluated by the method of leading-front.[43] The actual control random movement is 32 μm ± 3 SE of 10 separate experiments performed in duplicate.

Chemotaxis. In order to study the potential chemotactic activity, each peptide was added to the lower compartment of the chemotaxis chamber. Peptides were diluted from a stock solution (DMSO; 10^{-2} M) with KRPG containing 1 mg/mL of bovine serum albumin (Orha Behringwerke, Germany) and used at concentrations ranging from 10^{-12} to 10^{-5} M. Data were expressed in terms of chemotactic index, which is the ratio: (migration toward test attractant minus migration toward the buffer)/migration toward the buffer; the values are the mean of six separate experiments performed in duplicate. Standard errors are in the 0.02–0.09 chemotactic index range.

Table 4. Crystal data of *N*-Boc-Adt-OMe (**5**)

Empirical formula	$C_{10}H_{17}NO_4S_2$
Formula weight	279.4
Crystal system	Monoclinic
a	6.081(2) Å
b	19.783(5) Å
c	11.358(2) Å
β	91.49(2)°
V	1365.8(6) $Å^3$
Space group	$P2_1/n$
d_c	1.36 g cm^{-3}
Z	4
F(000)	592
λ(Cu-K_α)	1.5418 Å
μ(Cu-K_α)	3.46 mm^{-1}
Crystal size	0.75×0.10×0.05 mm
$2\theta_{max}$	124°
Reflections with $I > 3\sigma(I)$	1310
No. variables	154
R, R_w	0.057, 0.066
Full-matrix least-squares weight	sin ϑ/λ
S	0.9
Max and min residual electron density	0.3*to*−0.2 e/$Å^3$

Enzyme assay. The release of neutrophil granule enzymes was evaluated by determination of lysozyme activity, modified for microplate-based assays. Cells, 3×10^6/well, were first incubated in triplicate wells of microplates with 5 μg/mL cytochalasin B at 37 °C for 15 min and then in the presence of each peptide in a final concentration of 10^{-9}–2×10^{-5} M for further 15 min. The plates were then centrifuged at 400*g* for 5 min and the lysozyme was quantified nephelometrically by the rate of lysis of cell wall suspension of *Micrococcus lysodeikticus*. The reaction rate was measured using a microplate reader at 465 nm. Enzyme release was expressed as a net percentage of total enzyme content released by 0.1% Triton X-100. Total enzyme activity was 85 ± 1 μg/1×10^7 cells/min. The values are the mean of five separate experiments done in duplicate. Standard errors are in 1–6% range.

Superoxide anion (O_2^-) production. The superoxide anion was measured by the superoxide dismutase-inhibitable reduction of ferrycytochrome *c* (Sigma, St. Louis, MO, USA) modified for microplate-based assays. Tests were carried out in a final volume of 200 μL containing 4×10^5 neutrophils, 100 nmol cytochrome c (Sigma) and KRPG. At zero time, different amounts (10^{-9}–2×10^{-5} M) of each peptide were added and the plates were incubated into a microplate reader (Ceres 900, Bio-Tek Instruments, Inc.) with the compartment temperature set at 37 °C. Absorbance was recorded at wavelengths of 550 and 465 nm. Differences in absorbance at the two wavelengths were used to calculate nmol of O_2^- produced using an absorptivity for cytochrome *c* of 18.5 mM^{-1}/cm. Neutrophils were incubated with 5 μg/ml cytochalasin B (Sigma) for 5 min prior to activation by peptides. The points are the mean of five separate experiments done in duplicate. Standard errors are in the 0.1–4 nmols O_2^- range.

Statistical analysis. The non-parametric Wilcoxon test was used in the statistical evaluation of differences between groups.

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