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Synthesis and biological evaluation of new amino acids structurally related to the antitumor agent acivicin

Paola Conti [a,*], Gabriella Roda [a], Helena Stabile [b], Maria Antonietta Vanoni [c], Bruno Curti [b], Marco De Amici [a,*]

[a] *Istituto di Chimica Farmaceutica, Università di Milano, viale Abruzzi 42, Milano 20131, Italy*
[b] *Dipartimento di Fisiologia e Biochimica Generali, Università di Milano, Via Celoria 26, Milano 20131, Italy*
[c] *Dipartimento di Scienze Chimiche, Fisiche e Matematiche, Università dell'Insubria, Via Valleggio 11, Como 22100, Italy*

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Abstract

A set of racemic conformationally constrained analogues of the antitumor antibiotic acivicin (+)-**1** has been prepared through a strategy based on 1,3-dipolar cycloaddition of bromonitrile oxide to suitable dipolarophiles. The bromo analogue (**2**) of acivicin was also synthesized and tested as a reference compound, together with its stereoisomer **3**. The antitumor properties of novel amino acids **4**–**7** were evaluated in vitro against human tumor cell lines. Their efficacy to inhibit glutamate synthase (GltS) from *Azospirillum brasilense* was also assayed. None of the studied compounds, but **2**, showed significant activity.
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Keywords: Amino acids; Antitumor agent; Acivicin

1. Introduction

Acivicin [(α*S*,5*S*)-α-amino-3-chloro-4,5-dihydro-isoxazol-5-yl acetic acid, AT-125] (+)-**1** (Fig. 1) is an antitumor agent known to inhibit cell growth [1]. Acivicin was first isolated as a fermentation product of *Streptomyces sviceus* [1], and it was shown to behave as a glutamine antimetabolite [2], able to inhibit a number of L-glutamine-dependent amidotransferases, e.g. cytosine triphosphate synthetase (CTP-synthetase), xanthosine monophosphate aminase (XMP-aminase), carbamoyl-phosphate synthetase II [3], anthranilate synthase, asparagine synthetase and glutamate synthase (GltS) [4]. Moreover, acivicin behaves as a potent γ-glutamyl transpeptidase (γ-GT) inhibitor [5].

A number of experimental evidences suggested that acivicin could be potentially useful in the treatment of certain tumors, i.e. myeloid leukemia [6,7]. However, a number of severe side effects associated with the use of acivicin emerged during clinical trials, e.g. myelotoxicity and damages of the central nervous system [3]. Therefore, acivicin represents an excellent lead for the design of novel structurally related derivatives with an improved selectivity and a reduced toxicity. We started an investigation on the structure–activity relationship of acivicin through the design and synthesis of a set of conformationally constrained analogues of acivicin.

The novel derivatives are characterized by the presence in their structure of the 3-bromo-Δ^2-isoxazoline moiety, at variance with acivicin, which contains the 3-chloro-Δ^2-isoxazoline portion. Previous investigations [8] demonstrated that such a structural modification does not significantly affect the pharmacological profile of the compounds. As a matter of fact, when assayed in vivo for antitumor activity in the L1210 murine leukemia test at a dose of 8 mg/kg (±)-**1** produced a *T*/*C* (%) equal to 185 (*T* being the median survival time of treated animals and *C* being the median survival time of control animals) versus a value of 169 displayed by its bromo analogue (±)-**2** [8]. Accordingly, due to its easier

* Corresponding authors.
E-mail address: paola.conti@unimi.it (P. Conti).

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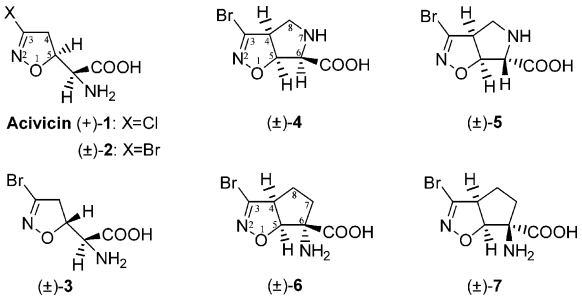


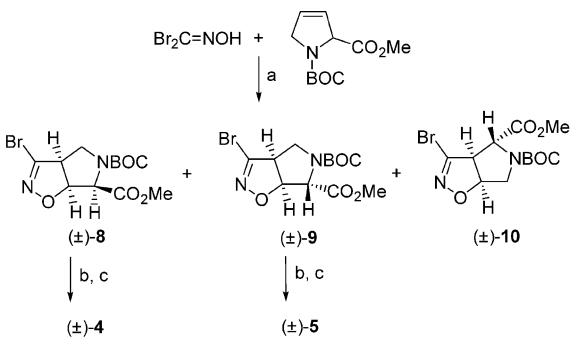
Fig. 1. Chemical structure of model and target compounds.

and safer synthetic access [8–10], (±)-**2** was prepared and used as the reference compound.

This paper deals with the synthesis of bicyclic derivatives (±)-**4**–(±)-**7** (Fig. 1), which represent different locked conformations of (±)-**2**. The antitumor activity of (±)-**2**, its stereoisomer (±)-**3** [8–10] and novel derivatives (±)-**4**–(±)-**7** was evaluated at the National Cancer Institute (Bethesda, MD) in a panel of in vitro disease-oriented primary antitumor tests. Furthermore, the ability of the newly synthesized compounds to inhibit *Azospirillum brasilense* GltS, a well-characterized enzyme belonging to L-glutamine-dependent amidotransferase class [11,12] was determined.

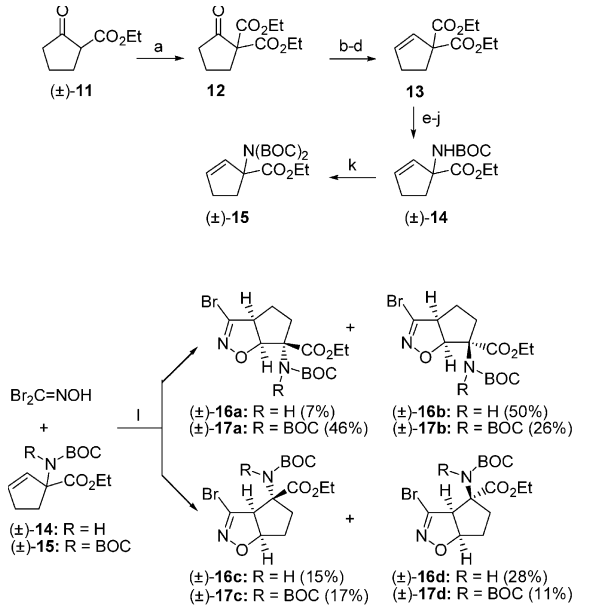
2. Chemistry

The synthesis of 3-bromo-Δ^2-isoxazolinylprolines (±)-**4** and (±)-**5** was accomplished according to the reaction sequence illustrated in Scheme 1. As previously reported [13], 1,3-dipolar cycloaddition of bromonitrile oxide to racemic *N*-BOC-3,4-didehydroproline methyl ester yielded three out of four possible stereoisomers in similar amounts. The separation of the cycloadducts was rather laborious, since a column chromatography of the reaction mixture gave two fractions containing pure (±)-**8** and a mixture of (±)-**9** and (±)-**10**. Fortunately, cycloadduct (±)-**9** could be separated from (±)-**10** by



a: $NaHCO_3$/AcOEt; b: 4N HCl, reflux; c: Amberlite IR-120 plus, 10% Py (aq.)

Scheme 1.



a: EtMgBr, ClCOOEt, THF; b: $NaBH_4$, EtOH; c: $MeSO_2Cl$, TEA; d: DBU, toluene, Δ; e: 1N NaOH (1 eq.), EtOH; f: TEA, ClCOOEt, acetone; g:NaN_3, H_2O; h: benzene, Δ; i: HCl, THF-H_2O; j: $(BOC)_2O$, TEA, CH_2Cl_2; k: $(BOC)_2O$, DMAP, THF, Δ; l: $NaHCO_3$, AcOEt

Scheme 2.

fractional crystallization. Intermediates (±)-**8** and (±)-**9** were heated at reflux with 4 N HCl followed by ion exchange chromatography to yield final amino acids (±)-**4** and (±)-**5** in a pure form.

Following a similar approach, amino acids (±)-**6** and (±)-**7** (Fig. 1) were prepared through 1,3-dipolar cycloaddition of bromonitrile oxide to cyclopentene derivatives (±)-**14** or (±)-**15** (Scheme 2). Dipolarophiles (±)-**14** and (±)-**15** were synthesized according to the reaction sequence reported in Scheme 2. Based on a procedure previously reported in the literature [14], commercially available 2-oxo-cyclopentane-carboxylic acid ethyl ester (±)-**11** was transformed into intermediate diester **12**. The keto derivative **12** was reduced to the related secondary alcohol with sodium borohydride and then transformed into the corresponding methanesulfonate, which was subsequently reacted with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) at reflux in toluene to yield alkene **13**. This intermediate was treated with 1 equiv. of sodium hydroxide to afford the corresponding monoacid, which was then submitted to the Curtius rearrangement. The emerging primary amino group was reacted with di-*tert*-butyl dicarbonate, under a standard procedure, to give *N*-BOC derivative (±)-**14**. The transformation of alkene (±)-**14** into (±)-**15** needed harsher conditions, since it was necessary to reflux a THF solution of (±)-**14** and excess di-*tert*-butyl dicarbonate in the presence of a catalytic amount of 4-(dimethylamino)pyridine (DMAP).

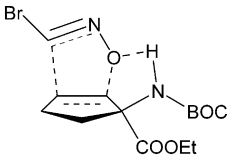
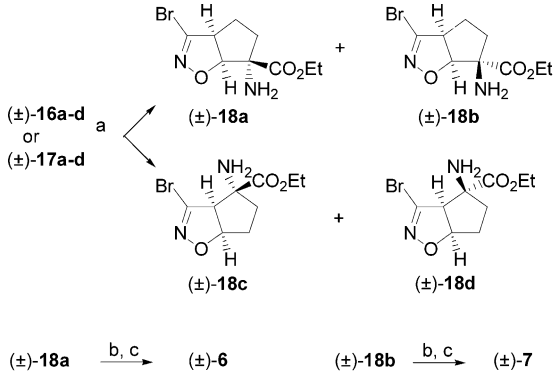


Fig. 2. Representation of the transition state leading to (±)-**16b**.

It is worth pointing out that the pericyclic reaction produces all four possible stereoisomers **16a/16b/16c/16d** in a relative ratio of 7:50:15:28. By far, the major stereoisomer of the reaction mixture is (±)-**16b**. As already observed in similar instances [15–17], the transition state leading to (±)-**16b** is stabilized by a hydrogen bond between the NHBOC group of the dipolarophile and the negatively charged oxygen of the nitrile oxide, as depicted in Fig. 2.

In order to reverse the stereopreference observed with dipolarophile (±)-**14**, we carried out the corresponding 1,3-dipolar cycloaddition of bromonitrile oxide to alkene (±)-**15**. In this case, we caused both an increase in the bulkiness of the carbamate group and the removal of the hydrogen bond interaction. The pericyclic reaction gave a mixture of four cycloadducts **17a/17b/17c/17d** in a relative ratio of 46:26:17:11 (Scheme 2). As expected, (±)-**17b** (26%) is not any longer the major stereoisomer of the reaction mixture, but is overcome by (±)-**17a** (46%).

In both cases, the separation of four cycloadducts could only be performed after their conversion into the corresponding primary amines (±)-**18a**–(±)-**18d**. As shown in Scheme 3, the treatment of the crude reaction mixtures (±)-**16a**–(±)-**16d** and (±)-**17a**–(±)-**17d** with a dichloromethane solution of trifluoroacetic acid afforded a mixture of amino esters (±)-**18a**–(±)-**18d** in quantitative yield, which were separated by column chromatography. Intermediates (±)-**18a** and (±)-**18b** were converted into final zwitterionic amino acids (±)-**6** and (±)-**7** by hydrolysis with 1 N NaOH, followed by ion exchange chromatography.



a: 30% TFA, CH_2Cl_2, b: 1N NaOH; c:Amberlite IR-120 plus, 10% Py (aq.)

Scheme 3.

For analytical purposes, amino esters (±)-**18a**–(±)-**18d** were separately reconverted under standard conditions (see Section 4) into the corresponding mono-BOC derivatives (±)-**16a**–(±)-**16d** and di-BOC derivatives (±)-**17a**–(±)-**17d**. The relative percentages of stereoisomers (±)-**16a**–(±)-**16d** and (±)-**17a**–(±)-**17d**, reported in Scheme 2, were obtained by HPLC analysis of the two crude reaction mixtures.

The structure to the cycloadducts was assigned on the basis of the ^{1}H NMR spectrum of the corresponding amines (±)-**18a**–(±)-**18d**. The multiplicity of H-5, the most deshielded proton, is diagnostic for the assignment of the regiochemistry since it resonates as a doublet in cycloadducts (±)-**18a** and (±)-**18b**, and as a multiplet in cycloadducts (±)-**18c** and (±)-**18d**. The structure to the couples of stereoisomers **18a/18b** and **18c/18d** was assigned by taking into account the upfield shift of proton H-4 and H-5, respectively, observed in compounds (±)-**18a** and (±)-**18c**. Such a shielding effect is due to the influence of the spatially close amino group. As a matter of fact, H-4 of (±)-**18c** resonates at 3.42 ppm versus 4.03 ppm observed in derivative (±)-**18d**. The same considerations hold true for H-5 in derivatives (±)-**18a** and (±)-**18b** (4.73 ppm versus 5.08 ppm).

3. Results and discussion

Amino acids (±)-**2**, (±)-**3**, (±)-**4**, (±)-**5**, (±)-**6** and (±)-**7** were submitted to the National Cancer Institute Developmental Therapeutics Program, which consists in an in vitro disease-oriented primary antitumor screening [18,19]. This investigation utilizes 60 different human tumor cell lines, representative of leukemia, melanoma and cancers of the lung, colon, brain, ovary, breast, prostate and kidney. In agreement with previously reported data, our reference compound (±)-**2** showed cell growth inhibition. According to the protocol, the activity is expressed using three parameters: GI_{50}, TGI and LC_{50}, which indicate the $\log_{10}$ of the molar concentration that produces, respectively, 50% cell growth inhibition, total cell growth inhibition and 50% cell death. The average values displayed by compound (±)-**2**, referred to all cell lines, are $GI_{50} = -4.60$, $TGI = -4.01$ and $LC_{50} = -4.00$. These values are comparable with those reported for acivicin ($GI_{50} = -4.74$, $TGI = -4.00$ and $LC_{50} = -4.00$) [20]. Unfortunately, none of the novel compounds (±)-**3**–(±)-**7** showed comparable levels of cell growth inhibition, since they exhibited values of GI_{50}, TGI and $LC_{50} \geq -4.00$.

In parallel, the set of new compounds has been tested in a relatively simple assay, based on the inhibition and inactivation of *A. brasilense* GltS. It has been reported that acivicin behaves as an inhibitor of several amidotransferases, by competing with glutamine for the same

binding site [4]. Incubation of these enzymes with acivicin leads to a time-dependent loss of activity, due to the formation of a covalent addition product with the catalytically essential cysteine residue located in the glutamine amidotransferase (GAT) site of these enzymes [12]. Acivicin was also shown to act as an irreversible inhibitor of the *Escherichia coli* GltS ($K_{inact} = 0.57$ mM; $k_{inact} = 0.19$ min^{-1}) [4], and to compete with L-glutamine ($K_i = 0.56$ mM) for the same binding site [4]. Based on the sequence homology among GltSs [11] as well as the structural similarity between GAT domain of GltSs and that of other Type-II amidotransferases [11,12,21], we tested the activity of acivicin bioisostere (±)-**2** as an inhibitor/inactivator of the well-characterized *A. brasilense* GltS. Compound (±)-**2** behaved as an inhibitor of the enzyme, competitive with L-glutamine, with a $K_i = 3.94 \pm 0.44$ mM. This value is similar to that obtained for acivicin with *E. coli* GltS [4], if we take into account that compound (±)-**2** was tested as a racemate, whereas the value reported for acivicin refers to the eutomer (+)-**1** only. Furthermore, compound (±)-**2** was able to inactivate *A. brasilense* GltS with an observed rate of approximately 0.02 min^{-1} when the enzyme (3 μM) was incubated with compound (±)-**2** (1 mM) at 25 °C. The L-glutamine analogue L-methionine sulfone, which binds to GltS with high affinity ($K_i \approx 2.5$ μM) [22], fully protects the enzyme, demonstrating that compound (±)-**2** inactivates the enzyme through its interaction with the glutamine binding site.

None of the newly synthesized compounds [(±)-**3**–(±)-**7**] exhibited any inhibitory effect of GltS catalytic activity nor inactivated the enzyme upon incubation for several hours at 25 °C. These results match those obtained in the in vitro antitumor tests. As a consequence, the evaluation of inhibition or inactivation of *A. brasilense* GltS could represent a convenient model for a pre-screening of new molecules designed as acivicin analogues. Furthermore, since the three-dimensional structures of several GATs have been solved [12], including those of *A. brasilense* [21] and *Synechocystis* GltSs [23], the rational design of novel acivicin analogues is now feasible.

The results of the present investigation evidence that the conformation of the side chain of acivicin is a critical requirement for its binding to the target enzymes. Indeed, the modification of the torsional angles between the α-amino acid group and the isoxazoline ring brings about the loss of any biological activity. Thus, in the design of new antitumor agents acting as acivicin analogues, the only "allowed" structural modifications appear to be those that do not alter the conformational requirements of the parent compound. On the basis of these results, we have now designed a set of novel amino acids, in which the nature of the heterocyclic ring of model compound (±)-**2** has been modified.

4. Experimental

4.1. Material and methods

Dibromoformaldoxime [8], compounds (±)-**2**, (±)-**3** [8–10], (±)-**8**–(±)-**10** [13] and **12** [14] were prepared according to literature procedures. ^{1}H NMR spectra were recorded with a 300 MHz Varian spectrometer in $CDCl_3$ or D_2O at 20 °C; the signal assignments are the results of a combination of 1D and 2D COSY. Chemical shifts (δ) are expressed in ppm and coupling constants (J) in hertz. HPLC analyses were performed on a LiChrospher Si 60 Merck column, using a Jasco PU-980 pump equipped with a UV–Vis detector Jasco UV-975. TLC was performed on commercial silica gel 60 F254 aluminum sheets; spots were further evidenced by spraying with dilute alkaline potassium permanganate solution or with ninhydrin. Melting points were determined with a capillary method on a Büchi B 540 apparatus and are uncorrected. Liquid compounds were characterized by the oven temperature for Kugelrohr distillations. Microanalyses of new compounds agreed with theoretical values of ±0.3%.

4.1.1. Synthesis of amino acids (±)-4 and (±)-5

Compound (±)-**8** [13] (300 mg, 0.86 mmol) was refluxed with 4 N HCl (9 ml) for 3 h. After evaporation of the solvent, the residue was dissolved in water and submitted to cation exchange chromatography, using Amberlite IR-120 plus. The acidic solution was slowly eluted onto the resin, and then the column was washed with water until the pH was neutral. The compound was eluted off the resin with 10% aqueous pyridine, and the product-containing fractions (detected with ninhydrin stain on a TLC plate) were combined and concentrated under vacuum to give (±)-**4** (129 mg, 0.55 mmol) in 64% yield.

(±)-(3a*S*,6*R*,6a*S*)-3-Bromo-4,5,6,6a-tetrahydro-3a*H*-pyrrolo[3,4-*d*]isoxazole-6-carboxylic acid ((±)-**4**); m.p. (dec.) > 175 °C; colorless prisms from H_2O/EtOH; ^{1}H NMR (D_2O): 3.57 (dd, 1, $J = 8.1$, 12.9 Hz), 3.83 (d, 1, $J = 12.9$ Hz), 4.46 (dd, 1, $J = 8.1$, 9.3 Hz), 4.48 (d, 1, $J = 5.3$ Hz), 5.69 (dd, 1, $J = 5.3$, 9.3). Anal. ($C_6H_7BrN_2O_3$) C, H, N.

Compound (±)-**9** [13] (400 mg, 1.15 mmol) was refluxed with 4 N HCl (12 ml) for 3 h. After evaporation of the solvent, the residue was dissolved in water. Ion exchange chromatography (Amberlite IR-120 plus cation exchange: eluted with 10% pyridine in water) afforded (±)-**5** (190 mg, 0.81 mmol) in 70% yield.

(±)-(3a*S*,6*S*,6a*S*)-3-Bromo-4,5,6,6a-tetrahydro-3a*H*-pyrrolo[3,4-*d*]isoxazole-6-carboxylic acid ((±)-**5**); m.p. (dec.) > 172 °C; colorless prisms from H_2O/MeOH; ^{1}H NMR (D_2O): 3.68 (dd, 1, $J = 7.9$, 12.9 Hz), 3.84 (d, 1, $J = 12.9$ Hz), 4.48 (dd, 1, $J = 7.9$, 9.3 Hz), 4.51 (s, 1), 5.62 (d, 1, $J = 9.3$ Hz). Anal. ($C_6H_7BrN_2O_3$) C, H, N.

4.1.2. Synthesis of diethylcyclopent-2-ene-1,1-dicarboxylate (*13*)

To a stirred solution of **12** [14] (30.7 g, 134.7 mmol) in EtOH (300 ml), cooled at 0 °C, sodium borohydride (2.5 g, 67.3 mmol) was added portionwise. After stirring for 30 min at 0 °C, the reaction was quenched by a dropwise addition of 1 N HCl. After evaporation at reduced pressure of the volatiles, the residue was dissolved in water and extracted with dichloromethane. The organic extracts were dried over anhydrous sodium sulfate and concentrated. The crude material was submitted to column chromatography on silica gel (eluant: petroleum ether–ethyl acetate, 4:1) to give the corresponding alcohol derivative as a yellow oil (14.54 g; yield: 47%).

The above prepared alcohol (14.54 g, 63.21 mmol) was reacted with 1.5 equiv. of triethylamine (TEA) (13.1 ml, 94.8 mmol) and 1.5 equiv. of methanesulfonylchloride (7.4 ml, 94.8 mmol), in dichloromethane, at 0 °C. After stirring for further 30 min at room temperature (r.t.), 1 N HCl (6 ml) was added to the reaction mixture. The organic layer was separated and washed with an aqueous solution of $NaHCO_3$. After the usual work up, the crude material was purified by column chromatography on silica gel (eluant: petroleum ether–ethyl acetate, 9:1) to give 11.1 g of mesylate in 57% yield.

A solution of the mesylate obtained in the previous step (11.1 g, 36.04 mmol) and DBU (27 ml, 180.2 mmol) in toluene (90 ml) was heated at reflux for 48 h. After cooling at r.t. the reaction mixture was washed with 2 N HCl (2×30 ml) and water (1×30 ml). The organic phase was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude material was purified by column chromatography on silica gel (eluant: petroleum ether–ethyl acetate, 95:5) to give 6.88 g (90% yield) of **13** as a colorless liquid.

Diethylcyclopent-2-ene-1,1-dicarboxylate (**13**); colorless oil, b.p.: 70 °C/0.35 mbar; ^{1}H NMR ($CDCl_3$): 1.24 (t, 6, $J = 6.8$ Hz), 2.46 (m, 4), 4.18 (q, 4, $J = 6.8$ Hz), 5.82 (m, 1), 5.98 (m, 1). Anal. ($C_{11}H_{16}O_4$) C, H, N.

4.1.3. (±)-Ethyl-N-tert-butoxycarbonyl-1-amino-cyclopent-2-ene-1-carboxylate (*14*)

Alkene **13** (6.88 g, 32.5 mmol) was dissolved in EtOH (30 ml) and treated with 1 N NaOH (32.5 ml, 32.5 mmol) at r.t. for 15 h. After evaporation of the ethanol at reduced pressure, the aqueous layer was extracted with dichloromethane (3×10 ml) to remove unreacted **13**. The aqueous layer was then acidified with 2 N HCl and extracted with dichloromethane (3×15 ml). The organic phase was dried and evaporated to give 4.93 g of the corresponding monoacid of **13** which was not characterized but directly used in the next step.

The above-prepared monoacid derivative (4.93 g, 26.8 mmol) was dissolved in acetone (45 ml) and cooled at 0 °C. Triethylamine (4.48 ml, 32.2 mmol) was added at once followed by the dropwise addition of ethylchloroformate (3.44 ml, 36.1 mmol). The mixture was stirred at r.t. for 30 min, then cooled at 0 °C. A solution of sodium azide (2.63 g, 40.4 mmol) in water (5 ml) was slowly added and the mixture was stirred for 1 h at 0 °C. The progress of the reaction was monitored by TLC (eluant: petroleum ether–ethyl acetate, 4:1). After evaporation of the acetone at reduced pressure, the aqueous phase was extracted with Et_2O (2×10 ml) and the pooled organic extracts were dried and concentrated. The residue, containing acyl-azide, was dissolved in benzene (100 ml) and refluxed for 3 h to give the corresponding isocyanate. The progress of the reaction was monitored by TLC (eluant: petroleum ether–ethyl acetate, 9:1). When the conversion was complete, the solvent was evaporated under vacuum and the residue was dissolved in THF (100 ml) and reacted with 2 N HCl (100 ml) overnight. THF was evaporated at reduced pressure, the aqueous phase was extracted with dichloromethane (2×30 ml), then made alkaline with solid K_2CO_3. The product was extracted with ethyl acetate (5×30 ml). The combined organic extracts were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to give 2.05 g (50% yield) of the desired amino ester as a yellow oil.

A dichloromethane solution (30 ml) of the amino ester (2.05 g, 13.2 mmol) and TEA (2.76 ml, 19.8 mmol) was stirred and cooled at 0 °C. To the mixture, a solution of di-*tert*-butyl dicarbonate (4.3 g, 19.8 mmol) in CH_2Cl_2 (10 ml) was added dropwise and stirred at r.t. overnight. After treating with 1 N HCl, the organic layer was dried and concentrated under vacuum. The residue was purified by a silica gel column chromatography (eluant: petroleum ether–ethyl acetate, 9:1) to give alkene (±)-**14** as a yellow oil (2.9 g, 86% yield).

(±)-Ethyl-*N*-*tert*-butoxycarbonyl-1-amino-cyclopent-2-ene-1-carboxylate ((±)-**14**); ^{1}H NMR ($CDCl_3$): 1.26 (t, 3, $J = 7.2$ Hz), 1.43 (s, 9), 2.05 (m, 1), 2.55 (m, 2), 2.70 (m, 1), 4.19 (q, 2, $J = 7.2$ Hz), 5.18 (bs, 1), 5.67 (m, 1), 6.07 (m, 1). Anal. ($C_{13}H_{21}NO_4$) C, H, N.

4.1.4. (±)-Ethyl-N-di-tert-butoxycarbonyl-1-amino-cyclopent-2-ene-1-carboxylate (*15*)

To a solution of alkene (±)-**14** (1.6 g, 6.27 mmol) in THF (25 ml) under stirring was added DMAP (76 mg, 0.627 mmol) followed by the dropwise addition of a THF solution (5 ml) of di-*tert*-butyl dicarbonate (2.05 g, 9.4 mmol). After refluxing the mixture for 3 days, further BOC_2O was added (2.74 g, 12.54 mmol) and the reaction mixture was refluxed for additional 3 days. The progress of the reaction was monitored by TLC (eluant: petroleum ether–ethyl acetate, 9:1). After evaporation of the solvent, the crude material was purified by column chromatography on silica gel (eluant: petroleum ether–ethyl acetate, 95:5) to give alkene (±)-**15** as a yellow oil (1.96 g, 88% yield).

(±)-Ethyl-*N*-di-*tert*-butoxycarbonyl-1-amino-cyclopent-2-ene-1-carboxylate ((±)-**15**); ^{1}H NMR ($CDCl_3$): 1.24 (t, 3, $J = 6.9$ Hz), 1.47 (s, 18), 2.05 (m, 1), 2.53 (m, 2), 3.01 (m, 1), 4.16 (q, 2, $J = 6.9$ Hz), 5.72 (m, 1), 6.01 (m, 1). Anal. ($C_{18}H_{29}NO_6$) C, H, N.

*4.1.5. 1,3-Dipolar cycloaddition of bromonitrile oxide to (±)-**14***

To a solution of (±)-**14** (1.2 g, 4.7 mmol) in ethyl acetate (15 ml) was added dibromoformaldoxime (1.9 g, 9.4 mmol) and solid $NaHCO_3$ (3.5 g). The mixture was vigorously stirred for 3 days. The progress of the reaction was monitored by TLC (eluant: petroleum ether–ethyl acetate, 4:1). Water (10 ml) was added to the reaction mixture and the organic layer was separated and dried over anhydrous Na_2SO_4. The crude material, obtained after evaporation of the solvent, was purified by column chromatography on silica gel (eluant: petroleum ether–ethyl acetate, 85:15) to give 1.52 g (86% yield) of an unsplittable mixture of cycloadducts (±)-**16a**–(±)**16d**.

*4.1.6. 1,3-Dipolar cycloaddition of bromonitrile oxide to (±)-**15***

To a solution of (±)-**15** (1.9 g, 5.35 mmol) in ethyl acetate (20 ml) was added dibromoformaldoxime (2.17 g, 10.7 mmol) and solid $NaHCO_3$ (4 g). The mixture was vigorously stirred for 3 days. The progress of the reaction was monitored by TLC (eluant: petroleum ether–ethyl acetate, 9:1). After a further addition of 1 equiv. of dibromoformaldoxime (1.08 g, 5.35 mmol), the reaction was stirred for additional 3 days, until the disappearance of alkene. The crude material, obtained after the previously reported work up, was purified by column chromatography on silica gel (eluant: petroleum ether–ethyl acetate, 95:5) to give 1.76 g (69% yield) of an unseparable mixture of the cycloadducts (±)-**17a**–(±)-**17d**.

*4.1.7. Synthesis of intermediates (±)-**18a**, (±)-**18b**, (±)-**18c** and (±)-**18d***

The mixture of (±)-**16a**–(±)-**16d** (1.52 g, 4.03 mmol) was treated with a 30% dichloromethane solution of trifluoroacetic acid (10.3 ml) at 0 °C. The reaction mixture was stirred at r.t. until disappearance of the starting material (3 h). The volatiles were removed under vacuum and the residue was treated with a 10% K_2CO_3 solution (25 ml) and extracted with ethyl acetate (4 × 10 ml). The pooled organic extracts were dried over anhydrous Na_2SO_4 and concentrated under vacuum. The residue was chromatographed on silica gel (eluant: petroleum ether–ethyl acetate, 1:1) to give four fractions which were eluted in the following order: (±)-**18d** (298 mg), $R_F = 0.80$ (cyclohexane–ethyl acetate, 1:4); (±)-**18b** (530 mg), $R_F = 0.65$ (cyclohexane–ethyl acetate, 1:4); (±)-**18a** (75 mg), $R_F = 0.52$ (cyclohexane–ethyl acetate, 1:4); (±)-**18c** (160 mg), $R_F = 0.41$ (cyclohexane–ethyl acetate, 1:4).

In analogy, the mixture of (±)-**17a**–(±)-**17d** (1.76 g, 3.69 mmol) was treated under the same conditions to give (±)-**18d** (112 mg); (±)-**18b** (220 mg); (±)-**18a** (385 mg) and (±)-**18c** (90 mg).

Amino ester (±)-**18a**; ^{1}H NMR ($CDCl_3$): 1.30 (t, 3, $J = 7.4$ Hz), 1.58 (bs, 2), 1.69 (m, 1), 2.00–2.38 (m, 3), 3.89 (dd, 1, $J = 8.2$, 8.6 Hz), 4.23 (q, 2, $J = 7.4$ Hz), 4.73 (d, 1, $J = 8.6$ Hz).

Amino ester (±)-**18b**; ^{1}H NMR ($CDCl_3$): 1.26 (t, 3, $J = 7.3$ Hz), 1.58 (m, 1), 1.85 (bs, 2), 1.99 (m, 2), 2.11 (m, 1), 3.84 (m, 1), 4.18 (q, 2, $J = 7.3$ Hz), 5.08 (d, 1, $J = 9.5$ Hz).

Amino ester (±)-**18c**; ^{1}H NMR ($CDCl_3$): 1.29 (t, 3, $J = 7.4$ Hz), 1.62 (bs, 2), 1.69 (m, 1), 2.15–2.52 (m, 3), 3.42 (d, 1, $J = 8.2$ Hz), 4.20 (q, 2, $J = 7.4$ Hz), 5.31 (m, 1).

Amino ester (±)-**18d**; ^{1}H NMR ($CDCl_3$): 1.31 (t, 3, $J = 7.4$ Hz), 1.77 (bs, 2), 1.86 (m, 1), 2.20 (m, 3), 4.03 (d, 1, $J = 9.6$ Hz), 4.23 (q, 2, $J = 7.4$ Hz), 5.28 (m, 1).

*4.1.8. Conversion of (±)-**18a**–(±)-**18d** into the corresponding derivatives (±)-**16a**–(±)-**16d** and (±)-**17a**–(±)-**17d***

20 mg of each amino ester (±)-**18a**–(±)-**18d** was treated with 1.5 equiv. of TEA and 1.5 equiv. of di-*tert*-butyl dicarbonate in dichloromethane to give the corresponding *N*-BOC derivatives (±)-**16a**–(±)-**16d**, which were not isolated but directly used as standards for HPLC analysis (column: LiChrospher Si 60 Merck; eluant: petroleum ether–ethyl acetate, 85:15; flow: 0.5 ml/min; $\lambda = 254$ nm). Retention times: **16b**, 11.65 min; **16d**, 13.23 min; **16c**, 16.05 min; **16a**, 18.15 min.

In analogy, 20 mg of each amino ester (±)-**18a**–(±)-**18d** was dissolved in THF and refluxed in the presence of 1.5 equiv. of di-*tert*-butyl dicarbonate and a catalytic amount of DMAP to give the corresponding derivatives (±)-**17a**–(±)-**17d**, which were used as standards for HPLC analysis (column: LiChrospher Si 60 Merck; eluant: petroleum ether–ethyl acetate, 85:15; flow: 0.2 ml/min; $\lambda = 254$ nm). Retention times: **17c**, 20.35 min; **17d**, 22.45 min; **17b**, 24.36 min; **17a**, 27.90 min.

*4.1.9. Synthesis of amino acids (±)-**6** and (±)-7*

A solution of (±)-**18a** (380 mg, 1.37 mmol) in ethanol (3 ml) was treated with 1 N NaOH (1.5 ml). The solution was stirred at r.t. until TLC (eluant: petroleum ether–ethyl acetate, 1:4) showed the disappearance of the starting material (4 h). The solution was then made acidic (pH 2) with 2 N HCl and submitted to cation exchange chromatography, using Amberlite IR-120 plus. The acidic solution was slowly eluted onto the resin, then the column was washed with water until the pH was neutral. The compound was eluted off the resin with 10% aqueous pyridine, and the product-containing

fractions (detected with ninhydrin stain on a TLC plate) were combined and concentrated under vacuum to give 157 mg (46%) of (±)-**6**, which was crystallized from water–ethanol as white prisms.

(±)-(3a*S*,6*S*,6a*S*)-6-Amino-3-bromo-4,5,6,6a-tetrahydro-3a*H*-cyclopenta[*d*]isoxazole-6-carboxylic acid ((±)-**6**): $R_F = 0.48$ (*n*-butanol–water–acetic acid, 60:25:15); m.p. (dec.) > 180 °C; ^{1}H NMR (D_2O): 2.01 (m, 1), 2.15–2.42 (m, 3), 4.20 (t, 1, $J = 8.5$ Hz), 5.14 (d, 1, $J = 8.5$ Hz). Anal. ($C_7H_9BrN_2O_3$) C, H, N.

The above-reported protocol applied to (±)-**18b** (500 mg, 1.8 mmol) gave 237 mg (yield: 53%) of (±)-**7**, which was crystallized from water–ethanol as white prisms.

(±)-(3a*S*,6*R*,6a*S*)-6-Amino-3-bromo-4,5,6,6a-tetrahydro-3a*H*-cyclopenta[*d*]isoxazole-6-carboxylic acid ((±)-**7**): $R_F = 0.58$ (*n*-butanol–water–acetic acid, 60:25:15); m.p. (dec.) > 174 °C; ^{1}H NMR (D_2O): 1.84 (m, 1), 2.04–2.40 (m, 3), 4.22 (t, 1, $J = 8.8$ Hz), 5.25 (d, 1, $J = 8.8$ Hz). Anal. ($C_7H_9BrN_2O_3$) C, H, N.

4.2. Biological testing

4.2.1. Anticancer screening

Evaluation of anticancer activity of compounds (±)-**2**–(±)-**7** was performed at the National Cancer Institute (NCI) of Bethesda, MD, following the well-known in vitro disease-oriented antitumor screening program [18,19], which is based upon the use of multiple panels of 60 human tumor cell lines against which the compounds under study were tested at 10-fold dilutions of five different concentrations, ranging from 10^{-4} to 10^{-8} M. The percentage growth was evaluated spectrophotometrically versus controls not treated with test agents. A 48-h continuous drug exposure protocol was followed and a sulfurhodamine B (SRB) protein assay was used to estimate cell viability or growth.

4.2.2. Biochemical methods

Recombinant *A. brasilense* GltS was produced, purified and assayed as described [22]. Steady-state kinetic measurements were carried out at 25 °C in 50 mM Hepes–KOH buffer, pH 7.5. The inhibitory effect of acivicin analogues was determined by assaying enzyme activity in the presence of varying concentrations of L-glutamine, 2-oxoglutarate or NADPH and increasing concentrations of the compound under analysis. To test GltS inactivation, the enzyme (3 μM) was incubated in 25 mM Hepes–KOH buffer, pH 7.5, 1 mM EDTA, 10% glycerol, in the presence of the compound under analysis (1 mM) for up to 8 h. At different times after addition of the potential inactivator, enzyme activity was measured upon dilution in reaction mixtures containing saturating concentrations of the enzyme substrates [22].

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